Role of seminal fluid in sexual transmission of HIV-1

Thesis

UNRESTRICTED

ROLE OF SEMINAL FLUID IN SEXUAL TRANSMISSION OF HIV-1

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Chapter 2: Materials and Methods

ABSTRACT

This study analysed the reservoir of HIV-1 in semen and the effects of seminal plasma on the functional and phenotypic characteristics of dendritic cells (DCs), both in relation to immunoregulatory capabilities and susceptibility to infection with HIV. The reservoir of HIV-1 in semen was defined in order to assess the feasibility of 'sperm washing' as a means of reducing the risk of transmission in HIV-1-discordant couples.

The fact that neither viral RNA or DNA could be detected in spermatozoa and the lack of expression of CD4 both at the protein and mRNA levels suggests that spermatozoa are not susceptible to HIV-1 infection. Viral RNA was detected in seminal plasma and both RNA and DNA were detected in non-sperm cells. As a result of this study, sperm washing is a service that is now available at the Chelsea & Westminster Hospital to HIV-1-discordant couples. This service has resulted in several births and no seroconversions in any woman who has undergone the procedure.

The effects of seminal plasma on the phenotype and function of monocyte-derived DCs were investigated. Seminal plasma had suppressive effects on the allostimulatory capacity of DCs and such effects appeared to be due to down-regulation of co-stimulatory molecule expression on these cells. The suppressive effect of seminal plasma was abrogated by the removal of lipids. However, prostaglandins on their own did not have suppressive effects on DC function, suggesting that other seminal components are required to induce the

observed induction of suppression. The suppressive effect of seminal plasma on the allostimulatory function of DCs was overcome with TNF-α, as did the presence of HIV. The expression of HIV co-receptors was assessed on DCs. TNF-α induced maturation of DCs as demonstrated by reduced expression of CCR5 and increased CXCR4. Seminal plasma also enhanced expression of CXCR4 and infection with X4 strains of HIV, but down-regulated CCR5 expression and infection with R5 strains of HIV. Therefore, the regulation of co-receptor expression by DCs was associated with their susceptibility to infection with a HIV strain displaying the corresponding co-receptor usage.

In summary, semen is an important vehicle for transmission of HIV. Seminal plasma was demonstrated to have profound effects on DC phenotype, maturation and function. Such alterations would influence the outcome, in terms of HIV transmission, of the interaction between the DC and HIV-1 at the mucosal surface.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BFA	Brefeldin A
CAF	$CD8^+$ T-lymphocyte anti-viral T cells
CMV	Cytomegalovirus
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DAF	Decay accelerating factor
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
EBS	Earl's balanced salt
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ELC	EBV-induced molecule 1 ligand chemokine
ELISA	Enzyme linked immunosorbent assay
FcR	Fc receptor
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FDC	Follicular dendritic cell
GM-CSF	Granulocyte-monocyte-colony stimulating factor
gp	glycoprotein

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPC	IFN-α producing cell
IVF	*In vitro*-fertilisation
L	Ligand
LAV	lymphadenopathy-associated virus
LC	Langerhans' cells
LFA	Leukocyte function antigen
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MDDC	$CD14^+$ monocyte-derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompartibility complex
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
MMI	Medicol medium isotonic
M-tropic	Macrophage-tropic HIV strain (i.e. HIV strain preferentially infecting macrophages)
NSC	Non-sperm cell
NSI	Non-syncytium-inducing
OD	Optical density

p	Protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE	Prostaglandin E
PHA	Phytohaemaglutinin
PMA	Phorbol 12-Myristate 13-Acetate
PMN	Polymorphonuclear leukocytes
Q-red	Quantum red
R5	CCR5-utilising HIV strain
RANTES	Regulated upon activation of normal T cell-expressed and secreted
RPE-Cy5	Phycoerythrin cychrome 5
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SDF	Stroma cell-derived factor
SE	Standard error
SI	Syncytium-inducing or stimulation index (indices)
SIV	Simian immunodeficiency virus
SLC	Secondary lymphoid tissue
SPM	Seminal plasma
STD	Sexually transmitted disease
TAP	Transport associated protein

TGF	Transforming growth factor
TNF	Tumour necrosis factor
tRNA	transfer ribonucleic acid
T-tropic	T cell-tropic HIV strain (i.e. HIV strain preferentially infecting T-lymphocytes)
X4	CXCR4-utilising HIV strain

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PRESENTATIONS AND PUBLICATION ARISING FROM THIS THESIS

This work was presented in part at:

1. British Society for Immunology Annual Congress at Harogate, U.K. (1998)

   (Oral presentation by Kim LU)

   Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children.

   Kim LU, Johnson MR, Nelson MR, Sontag G, Gotch FM, Gilmour JW

2. British Society for Immunology Annual Congress at Harogate, U.K. (1998)

   (Oral presentation by Gilmour JW)

   Potential immunomodulatory effect of seminal plasma on dendritic cells.

   Kim LU, Gotch FM, Gilmour JW

3. British Society for Immunology Annual Congress at Harogate, U.K. (1999)

   (Poster presentation by Kim LU)

   The effect of semen on the phenotype and function of cultured dendritic cells.

   Kim LU, Gotch FM, Gilmour JW

Publication arising from this thesis:

Kim LU, Johnson MR, Nelson MR, Sontag G, Gotch FM, Gilmour JW.

Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children.

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I

INTRODUCTION

I.1. INTRODUCTION TO THE THESIS

The acquired immunodeficiency syndrome (AIDS) was first recognised as a new disease in 1981, with a report of *Pneumocystis carinii* pneumonia (PCP) (Masur *et al*, 1981; Gottlieb *et al*, 1981). In 1983, a retrovirus was isolated from the lymph node lymphocytes of a French homosexual patient with generalised hyperplasic lymphadenopathy and hence the virus was termed lymphadenopathy-associated virus (LAV) (Barre-Sinoussi *et al*, 1983; Montagnier *et al*, 1984). The following year, Gallo's group confirmed and extended this finding, linking this virus to the immunodeficiency syndrome and the virus was renamed human immunodeficiency virus (HIV) (Sarngadharan *et al*, 1985). In 1986, a second related HIV, HIV-2, was isolated from West Africa (Franchini *et al*, 1987). It has now been shown that the first apparent HIV infection was in 1959 in Africa (Zhu *et al*, 1998). Despite the nucleotide sequence homology between HIV-1 and HIV-2 being only 42% and their antigenic cross reactivity is restricted, the clinical consequence of infection with either virus is similar (Evans and Levy, 1989).

HIV is transmitted vertically from mother to infant, by sexual contact and by contact with infected blood (i.e. blood transfusion and in intravenous drug users). This thesis focuses on HIV-1 transmission via a sexual contact. Aim of this thesis

is to define the HIV reservoir in semen and to elucidate the effect of seminal plasma on dendritic cell (DC) function and also on the uptake of HIV-1 by DCs.

I.2. Epidemiology of HIV

The human immunodeficiency virus is a member of the lentivirus ("slow virus") genus of the *Retroviridae* family. HIV is grouped into two types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2), on the basis of serology and sequence analysis of molecularly cloned viral genomes. Phylogenetic studies have provided evidence that HIV-1 is closely related to a simian immundeficiency virus (SIV) that naturally infects the chimpanzee, *Pan troglodytes troglodytes*, (SIV_{cpz}) (Gao *et al*, 1999). HIV-2 is closely related to a SIV naturally infecting sooty mangabey monkeys, *Cercocebus atys*, (SIV_{smm}) (Hirsch *et al*, 1989; Gao *et al*, 1994). It is believed that zoonotic transfer of these non-human primate immunodeficiency viruses resulted in the emergence of HIV-1 and HIV-2 (Joag *et al*, 1996). HIV-1 is distributed throughout the world, whereas HIV-2 remains largely restricted to West Africa [Cameroon, Ivory Coast, Senegal (Joag *et al*, 1996; Miyazaki, 1995)]. It has been suggested that HIV-2 is less likely to be transmitted sexually than HIV-1 (reviewed by Li *et al*, 1998) and this may a reason for the more restricted geographic distribution of HIV-2. Both HIV-1 and 2 isolates display large sequence variations in the *env* gene. A classification scheme, based on *env* gene sequences, describes 10 subtypes/clades of HIV-1 (A to J; Main (M) group) and 5 subtypes of HIV-2 (A to E) (WHO network for HIV isolation and characterisation, 1994; Gao *et al*, 1994). For HIV-1, an outlier (O) group is found in Cameroon,

Gabon and Equatorial Guinea and a new (N) group was identified in two people in Cameroon in 1998 (Simon *et al*, 1998; Weiss and Wrangham, 1999). There is a high degree of intra-subtype diversification. Although mutation appears to be the major factor responsible for viral variation, recombination also occurs in individuals infected with viruses from different clades. It has been demonstrated that clade A and D recombination occurs frequently in Uganda (Gao *et al*, 1994; personal communication from Dr. D. Yirrel, Edinburgh University, UK). Some areas of the world harbour predominantly a single subtype (eg. subtype B is predominant in North America and Europe, subtype C in India and South Africa and subtype E in Thailand), whereas two or more subtypes may be prevalent in other populations. Many subtypes are frequently found in Sub-Saharan Africa and subtype B and C viruses are predominant in Latin America and Caribbean countries (Quinn, 1996; Essex, 1998). In no country, however, is a single subtype exclusive.

According to the recent data released by United Nations AIDS program (UNAIDS) and World Health Organisation (WHO) in 2000, approximately 15000 persons per day become infected, with more than 95% of new infections occurring in developing countries. About 36.1 million people worldwide are presently living with HIV. More than 10% of new infections are occurring in children under age of 15 and nearly half of new infections are in women. UNAIDS further estimated 3 million deaths from AIDS worldwide in 2000 and a total since the epidemic began of 21.8 million deaths. Each day, nearly 1200 children die as a result of AIDS and

a further 1600 are infected (AIDS epidemic update, December 2000, http://www.unaids.org).

I.3. HIV STRUCTURE AND REPLICATION

I.3.1. The structure of HIV

The HIV-1 virion has a diameter of about 110nm with 72 external viral transmembrane glycoproteins expressed in a host cell derived lipid bilayer envelope along with host class I and II MHC antigens (Nermit *et al*, 1993). The viral glycoprotein gp41 inserts into the lipid bilayer membrane and, with gp120, protrudes through to form the outer surface of the virus particle. The nucleoprotein core of the virion comprises two copies of the viral genomic RNA associated with tRNA molecules along with mature *gag* and *pol* protein products (fig.I.1a and b; Field *et al*, 1996; Karn, 1995[a]). The 9 kb RNA genome of HIV encodes for at least nine proteins (table I.1) which can be divided into three groups:

i) The major structural proteins *gag, pol,* and *env.*

ii) The regulatory proteins *tat, nef* and *rev.*

iii) The accessory proteins *vpr, vif* and *vpu* for HIV-1 or *vpx* for HIV-2.

The function of each protein is listed in table I.1.

Table I.1. The proteins encoded by HIV-1 and HIV-2 (Karn, 1995[b])

Gene		Protein	Function
Virion Proteins	*gag*	MA (p17; Matrix protein)	Membrane binding, nuclear localisation signal for the nuclear import of viral pre-integration complex, virus assembly
		CA (p24; Capsid protein)	Virus assembly, interaction with the Cyclophilin A required for viral infectivity
		NC (p9; Nucleocapsid protein)	RNA binding, virus assembly
	pol	PR (p11; Protease)	Cleavage of viral polyproteins, virus maturation
		RT (p66/p51; Reverse transcriptase/ RNase H)	Virus replication (RNA-dependent polymerase and hydrolysis of RNA from RNA:DNA hybrids)
		IN (p32; Integrase)	Virus replication (insertion of linear ds viral DNA into the host cell chromosome)
	env	SS (gp120; Envelope protein)	CD4 and second co- receptor binding, viral infectivity
		TM (gp41; Transmembrane envelope glycoprotein)	Contains fusion domain at N-terminus: fusion of viral membrane with the plasma membrane during virus entry, anchor the protein in the lipid bilayer of the viral envelope
Accessory Proteins	*vpr*	Vpr (Viral protein R)	Translocation of pre-integration complex to nucleus (nuclear localisation signal), arrest cellular proliferation resulting in reduced lysis of HIV-1 infected cells by CTLs
	vif	Vif (Viral infectivity factor)	Facilitating viral internalisation or uncoating
	vpx	Vpx (Viral protein X)	Viral structural protein found in HIV-2 only
	vpu	Vpu (Viral Protein U)	Down-regulation of CD4 expression, envelope maturation and virus release, found in HIV-1 only
Regulatory proteins	*tat*	Tat (Trans-activator protein)	Stimulates transcriptional elongation
	rev	Rev (Regulator of viral expression)	Regulator of late gene expression, stimulates appearance of unspliced mRNA in cytoplasm
	nef	Nef (Negative factor)	Down regulates CD4, regulate T cell signalling pathway

I.3.2. The viral life cycle

The replication cycle is divided into an early and a late phase: each phase consists of sequential steps which involve specific interactions of viral proteins and nucleic acids with host factors. The early phase starts with the attachment to and entry of a virion in to a cell, reverse transcription of the viral RNA genome into a double stranded (ds) DNA copy catalysed by viral reverse transcriptase, and transport of the DNA copy to the nucleus followed by integration into the host cell genome at a random site in the chromosome. The late phase involves transcription and processing of viral RNA from the integrated proviral DNA template and translation of viral proteins followed by release of progeny virions from the cell.

i) Attachment and entry of the virion to a host cell

The envelope glycoprotein gp120 binds to the CDR2-like region of the CD4 receptor D1 domain with an affinity constant on the order of 1nM (Karn, 1995[a]; Dragic *et al*, 1992) and also binds to a second receptor (e.g. CCR5 or CXCR4; refer to I.4 for detail). It is hypothesised this binding induces conformational changes in the envelope protein which unmask the gp41 fusogenic domain, leading to fusion of viral and cell membranes (Callebaut *et al*, 1993; Benjouad *et al*, 1997). Fusion results in release of the core in to the cell cytoplasm, followed by removal of outer core proteins, exposing the viral nucleoprotein complex.

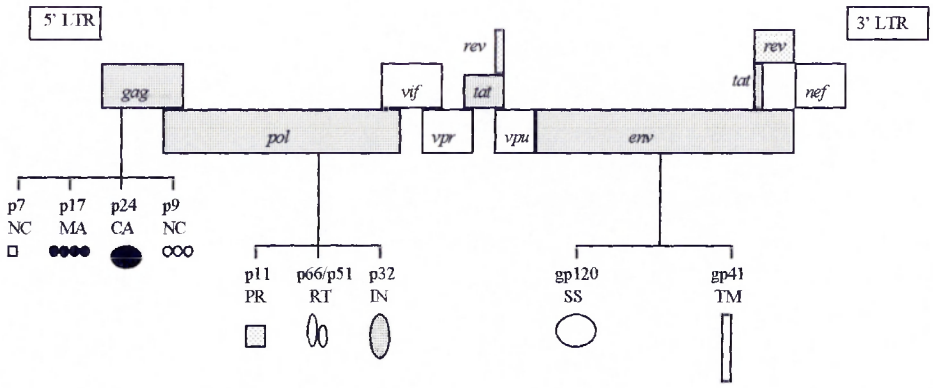
ii) Reverse transcription and integration

After entry of the core into the cytoplasm of a susceptible cell, viral RNA is reverse transcribed into a double-stranded (ds) DNA copy by viral reverse transcriptase (RT). HIV-1 particles contain two homologous genomic RNAs and $tRNA^{Lys}$ (Fig.I.1), which are used as primers for RNA-dependent DNA synthesis (Jiang *et al*, 1993; Ratner *et al*, 1985). The 18 3'-terminal nucleotides of $tRNA^{Lys}$, which form the anti-codon loop, are complementary to a primer-binding site within the viral RNA (Isel *et al*, 1993; Skripkin *et al*, 1996). This template-primer complex binds to viral RT. RT is a heterodimer consisting of p55 and p61 subunits and this heterodimeric form of RT is the result of carboxyl-terminal proteolytic cleavage of one subunit of the homodimer (p66/p66) (Chandra *et al*, 1986). HIV-1 RT is a multifunctional enzyme with three enzymatic activities: RNA-dependent DNA polymerase (RT), DNA-dependent DNA polymerase and RNase H. Both polymerase activities catalyse template-directed phophodiester bond formation in the 5' to 3' direction. The nucleoprotein complex containing the matrix protein and integrase is translocated to the nucleus. The transport signals for this appear to be encoded by the p17 matrix protein (Von Schwedler *et al*, 1994). It has also been shown that an intact *vpr* gene is required for nuclear entry of viral DNA (Heinzinger *et al*, 1994). The integration is mediated by the viral integrase which is responsible for DNA cleavage and strand exchange.

The type, activation and differentiation stage of the $CD4^+$ cell which has undergone fusion with a HIV virion influences the subsequent reverse

Fig. I.1. Genomic organisation of HIV (a) and the virion structure (b) (Fields *et al*, 1996; Karn 1995 [a])

(a)



NC = Nucleocapsid Protein

MA = Matrix Protein

CA = Major Capsid Protein

PR = Protease

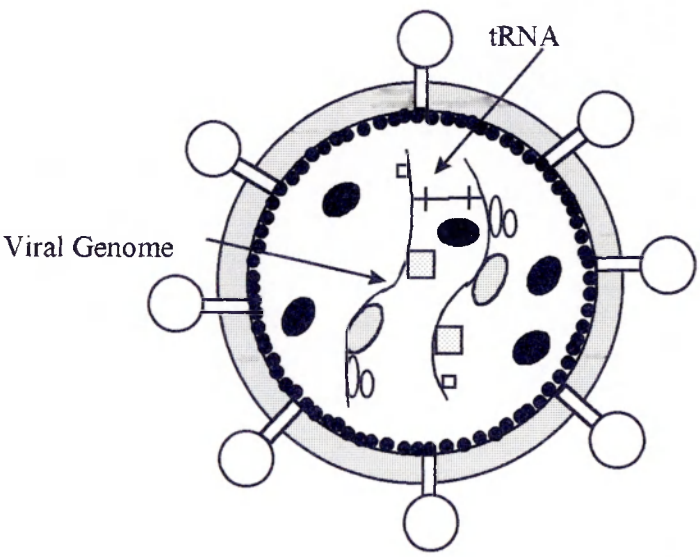
RT = Reverse Transcriptase

IN = Integrase

SS = Surface Subunit

TM = Transmembrane Subunit

(b)



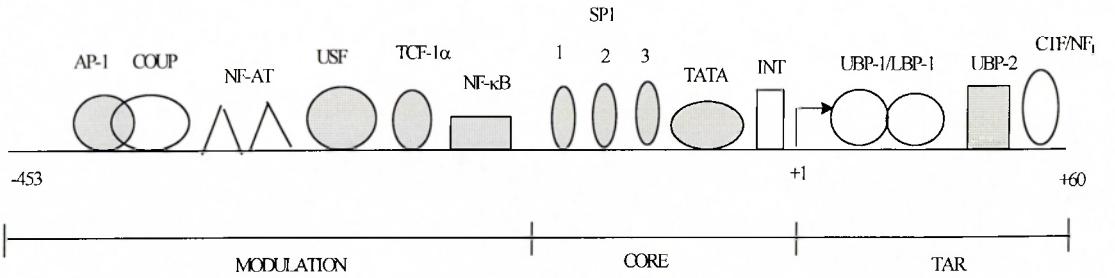
transcription and integration events. In terminally differentiated cells such as macrophages, reverse transcription results in complete proviral DNA copies that integrate in to the host DNA (Wienberg *et al*, 1991; Lewis *et al*, 1992). Viral gene expression and virion production may then follow integration in such cells. In T cells however, complete reverse transcription followed by integration and viral gene expression requires proliferation of the host cell (Zack *et al*, 1990). In non-proliferating T cells, HIV-1 may enter the cell with initiation of reverse transcription. Zack *et al* suggest that reverse transcription is incomplete and the resulting DNA is labile with an *in vitro* half-life of approximately 1 day. Others claim such extrachromosomal viral DNA may persist for weeks, with complete reverse transcription, integration and viral gene expression following subsequent activation of the cell (Stevenson *et al*, 1990).

iii) Expression of viral gene

Transcription of viral DNA and viral gene expression is controlled by both cellular and viral factors (Fig.I.2). The integrated provirus carries duplicate structures, called the long terminal repeats (LTR) at both 3'- and 5'-ends of the viral genome. The 5' LTR contains promoters for transcription and the 3' LTR provides a polyadenylation signal (Gaynor, 1992). As described above, viral gene expression in T cells requires proliferation of the host cell. Transcription of proviral DNA is greatly enhanced by host cell immune activation, cytokines [e.g. IL-1 (Kobayashi *et al*, 1989), IL-6 and TNF-α (Okamoto *et al*, 1989)] or gene products of other viruses (e.g. EBV, CMV, hepatitis B and herpesvirus).

These triggers can act to up-regulate host regulatory proteins, including the transcription factor NF-κB which binds to a site within the LTR, thereby enhancing viral transcription (Duh *et al*, 1989; Griffin *et al*, 1989; Tong-Starksen *et al*, 1987). *Tat* stimulates transcription from the viral LTR by interacting with a regulatory element in the 5' end, called the *trans*-activation-responsive region (TAR) (Sharp & Marciniak, 1989). This produces a modified transcription complex which is then able to transcribe the remainder of the HIV genome efficiently. *Tat* is also able to increase the density of RNA polymerases found downstream of the promoters and acts as an elongation factor. Transcription of the HIV genome results in three different mRNA species (Fig.I.3): 1) multiply spliced mRNAs which encode the viral regulatory proteins (*tat, rev*, and *nef*) and are the first mRNAs produced (Early mRNA), 2) later singly spliced mRNAs which encode *env* and accessory proteins (*vpu, vif*, and *vpr*) (Late mRNA) and 3) unspliced mRNAs which act both as the virion RNA and mRNA for g*ag-pol* (Late mRNA) (Kim *et al*, 1989). The early product *tat* (*trans*-activator of transcription) enhances transcription from the viral LTR by interacting with a regulatory element in the 5' LTR, called the *trans*-activation-responsive region (TAR) (Sharp & Marciniak, 1989). Accumulation of the other early product *rev* results in a switch to production of 'late mRNAs' while inhibiting 'early mRNA' production (Malim *et al*, 1989). *Rev* overcomes the RNA splicing mechanism by binding to a *rev*-responsive element (RRE) in the RNA resulting in production of singly and unspliced viral mRNAs encoding the structural proteins of the virus (g*ag, pol* and *env*) and full-length genomic RNA.

Fig. I.2. HIV-1 long terminal repeat DNA with potential binding sites for DNA and RNA binding proteins (reviewed by Ou *et al*, 1995)



COUP = ovalbumin upstream promoter

AP-1 = activator protein

NF-AT = nuclear factor of activated T cells

USF = upstream stimulatory factor

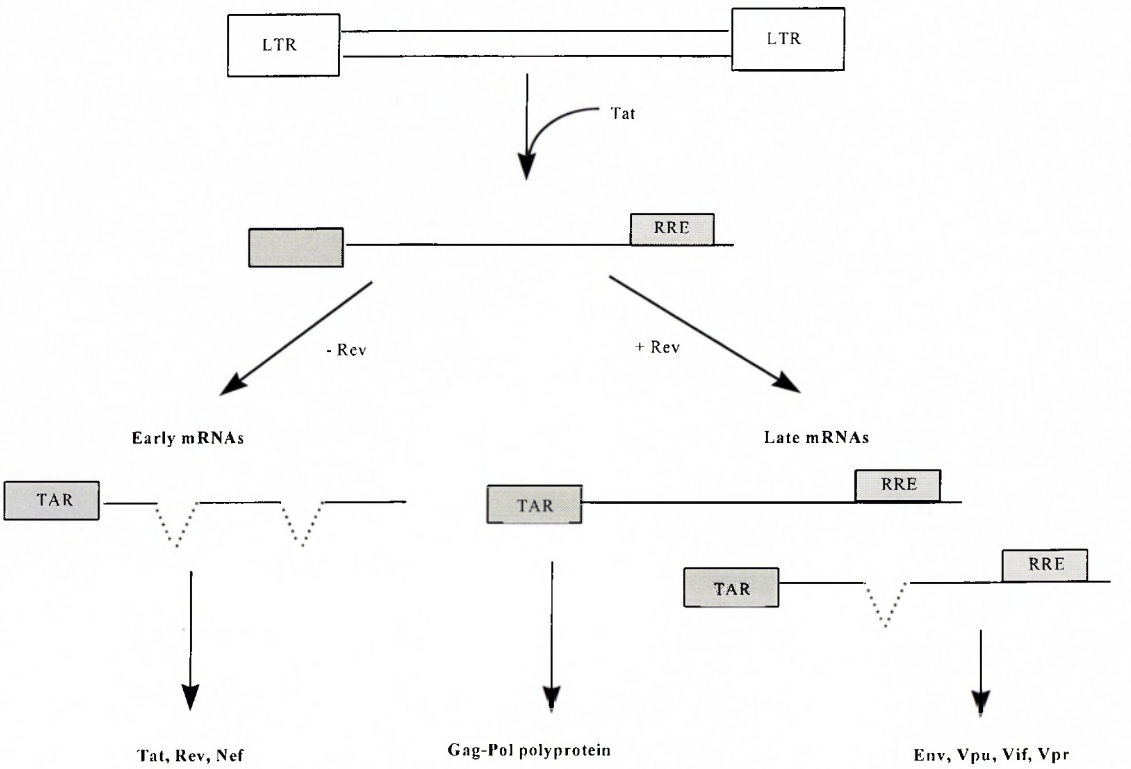
TCF-1α = T cell activator-1α

NF-κB = nuclear factor

UBP = untranslated binding protein

LBP-1 = binding protein-1

Fig. I.3. Regulation of HIV gene expression (reviewed by Karn, 1995[b])



Therefore, in host cell with minimal transcriptional activity, production of viral mRNAs and proteins would also be minimal, leading to latency of the integrated provirus.

iv) Assembly and release of virions

Env glycoproteins are synthesised as a gp160 precursor which host proteases cleave into amino-terminal gp120 and carboxy-terminal gp41. Gp41 contains a trans-membrane domain, spanning the host cellular membrane. Other viral structural components accumulate on the inner surface of the cytoplasmic membrane initiating the formation of an immature virus particle. The budding particles incorporate dimerized genomic RNA molecules, tRNA primers, the myristoylated *gag* and *gag-pol* precursor glycoproteins. HIV particles become enclosed by an envelope of host-cell lipid bilayer containing *env* glycoprotein. During or shortly after budding, viral particles mature. Maturation is accompanied by structural rearrangements leading to the assembly of a mature core structure (Luciw, 1996). Protease cleavage of the *gag* and *gag-pol* is essential in the maturation of retrovirus particles, as mutations in protease lead to production of non-infectious virus particles that contain uncleaved core proteins (Kohl *et al*, 1988; Katoh *et al*, 1985; Crawford and Goff, 1985).

HIV replication *in vivo* occurs continuously (Ho *et al*, 1995). Perselson *et al* (1996) developed a mathematical model to investigate HIV-1 dynamics *in vivo* using HIV-1 plasma viral load data collected after the administration of a potent HIV-1 protease inhibitor, Ritonavir. The average life-span of

productively infected cells was estimated to be 2.2 days and that of free virus in plasma was estimated to be 0.3 days. The average total HIV-1 production was estimated to be 10.3 x 10^9 virions per day and the average HIV-1 generation time (i.e. the time from release of a virion to release of a new generation of virions after infecting another cell) 2.6 days. The virus has a high mutation rate (3 x 10^{-5} mutation rate per nucleotide per replication cycle) since RT lacks proofreading activity and does not correct errors in DNA synthesis). Most mutations will confer a selective disadvantage to the virion, but some will be selected for, either by conferring resistance to anti-retroviral drugs or allowing evasion of the host immune response (Kusumi *et al*, 1992).

I.4. CELLULAR TROPISM OF HIV-1

HIV-1 infects cells that express cell surface CD4, including T cells, macrophages, DCs and brain microglia, via interaction of gp120 with CD4. CD4 was shown to be the primary receptor for HIV entry, as introduction of the CD4 gene rendered non-susceptible human cells susceptible to infection (Dalgleish *et al*, 1984). However, the requirement of an unknown second co-receptor was apparent, as human CD4 expression did not render rodent cells susceptible. In addition, HIV strains display cell tropisms that cannot be accounted for by CD4 being the single receptor for HIV. HIV-1 grown in T-cell lines *in vitro* can infect CD4 T cell lines and primary T cells, but not macrophages. However, many primary isolates of HIV infect primary T cells

and macrophages rather than T cell lines. These two types of viruses were initially referred to as T and M-tropic, respectively (Koyanagi *et al*, 1987; Fisher *et al*, 1988). M-tropic non-syncytium inducing isolates infect macrophages and primary T cells but not transformed T-cell lines, whereas T-tropic syncytium inducing isolates infect both primary T cells and T-cell lines but not macrophages.

I.4.1. Chemokine receptor usage by HIV-1 and their natural ligands

The HIV-1 co-receptors were shown to be receptors for chemokines and are responsible for binding the surface membranes of the host cell and the virus together to allow fusion of the membranes and viral entry. M-tropic strains utilise CCR5 primarily (Dragic *et al*, 1996; Alkhatib *et al*, 1996) and T-tropic strains utilise CXCR4 (Feng *et al*, 1996). These different virus strains are termed R5 strains or CCR5-utilising virus and X4 strains or CXCR4-utilising virus, respectively (Berger *et al*, 1998). Other chemokine receptors mediating HIV-1 entry include CCR2b, present on macrophages and CCR3 which is expressed on eosinophils and only at low levels on T cells, monocytes and DCs (Doranz *et al*, 1996; Rubbert *et al*, 1998). The *env* gene, particularly a region of gp120 that includes the V3 loop, determines T- versus M-tropism (Berger, 1997) with each type of isolate requiring different co-receptors. The co-receptors are either α or β-chemokine receptors (table I.2) which are seven transmembrane receptors from the large family of G protein coupled receptors which respond to chemokines, neurotransmitters and peptide hormones. The α-

chemokines (CXC chemokines) contain a single amino acid between the first and second N-terminal cysteine residues and they chemoattract and activate neutrophils primarily. The β-chemokines (CC chemokines) have adjacent cysteine residues and chemoattract and activate monocytes, lymphocytes and basophils. The natural chemokine ligands of these co-receptors inhibit or suppress infection of cells with HIV-1 by receptor competition. RANTES and macrophage inflammatory proteins (MIP-1α and MIP-1β), the natural ligands of CCR5, inhibit infection with M-tropic HIV-1 strains (Cocchi *et al*, 1995, Dragic *et al*, 1996). Stromal cell-derived factor-1 (SDF-1), the ligand of CXCR4, inhibits infection with T-tropic strains (Bleul *et al*, 1996; Oberlin *et al*, 1996). Similarly, exotaxin competes with HIV-1 for CCR3 (Choe *et al*, 1996), while monocyte chemotactic proteins (MCP-2, -3) compete with HIV-1 for CCR2 (Bisset *et al*, 1997; Schols *et al*, 1997). The macrophage-derived chemokine (MDC) inhibits a variety of HIV-1 strains irrespective of their tropism (Pal *et al*, 1997). It has also been demonstrated that the occupation of CCR5 and CXCR4 by their natural ligands results in the internalisation of these receptors, which may contribute to the suppressive effect of chemokines on HIV infectivity by reducing co-receptors densities (Amara *et al*, 1997).

The chemokines, RANTES, MIP-1α and MIP-1β were the first $CD8^+$ T cell derived factors that were identified to mediate HIV suppression (Cocchi *et al*, 1995). In addition to chemokines, $CD8^+$ T cells can release other soluble factors capable of suppressing viral replication, including cytotoxic molecules that mediate lysis of virus-infected cells such as IL-16 (Baier *et al*, 1995) and

as yet unidentified factors termed '$CD8^+$ T cell antiviral factor (CAF)'. IL-16 which is a natural ligand for CD4 was shown to inhibit HIV-1 replication in $CD4^+$ T cells, macrophages and DCs *in vitro* (Truong *et al*, 1999; Hermann *et al*, 1999). It has been demonstrated that filtered $CD8^+$ T-cell culture supernatants can suppress HIV-1 replication in naturally infected $CD4^+$ T cells without affecting $CD4^+$ T cell proliferation (Mackewicz and Levy, 1992; Brinchman *et al*, 1990; Mackewicz *et al*, 1994). Furthermore, Mackewicz and Levy (1992) have reported that asymptomatic individuals possess higher levels of CAF which are maintained over long periods of time. As disease progresses, CAF levels decline and are undetectable in AIDS patients (Mackewicz *et al*, 1994). Studies examining the effect of recombinant cytokines on HIV replication, the effect of anti-cytokine neutralising antibodies on CAF activity and by directly measuring the relative levels of known cytokines and chemokines in CAF-containing and non-CAF-containing culture supernatants, have indicated that CAF lacks identity to known cytokines (Mackewicz *et al*, 1994; Levy *et al*, 1996).

'Dual' tropism, HIV-1 isolates displaying both M- and T-tropism, is common among syncytium-inducing (SI) HIV strains. Such frequent dual receptor usage suggests that most SI isolates in fact contain a mixed population of SI and non-syncytium-inducing (NSI) quasi-species of virus capable of using both CCR5 and CXCR4. In addition, this 'dual' tropism of SI strains could suggest adaptation of HIV-1 to use CXCR4 during phenotypic evolution without losing the ability to use CCR5 (Zhang *et al.* 1996). However, a study

by Scarlatti *et al* (1997) argues against this concept. They have found a consistent pattern in the evolution of viral co-receptor usage and sensitivity to chemokine-mediated suppression in a longitudinal follow-up of children with progressive HIV-1 infection. Viral isolates obtained during the asymptomatic stages of HIV-1 infection mainly used CCR5 as a co-receptor and were inhibited by its natural ligands but not by SDF-1. In contrast, the majority of the isolates obtained after disease progression had acquired the ability to use CXCR4 and, in some cases, CCR3, while gradually losing CCR5 usage. Furthermore, all these latter isolates were resistant to β-chemokines and most were resistant to SDF-1. In children who progressed to AIDS without a shift to CXCR4 usage, all sequential isolates were CCR5-dependent but showed a reduced sensitivity to β-chemokines. Changes in the V3 domain of gp120 were associated with a loss of sensitivity to β-chemokines and a shift in co-receptor usage (Scarlatti *et al*, 1997).

[N.B. New nomenclature for chemokines listed in table I.2 below (Zlotnik and Yoshie, 2000): GRO-α/MGSA-α=CXCL1, GRO-β/MGSA-β=CXCL2, GRO-γ/MGSA-γ=CXCL3, ENA-78=CXCL5, NAP-2=CXCL7, IL-8=CXCL8, Mig=CXCL9, IP-10=CXCL10, SDF-1=CXCL12, MCP-1=CCL2, MIP-1α=CCL3, MIP-1β=CCL4, RANTES=CCL5, MCP-3=CCL7, MCP-2=CCL8, Eotaxin= CCL11, MCP-4=CCL13, ELC/MIP-3β=CCL19, MIP-3α=CCL20, Secondary lymphoid tissue chemokines (SLC)/6Ckine= CCL21]

Table I.2. Chemokine receptors and their natural ligands

Chemokine family	Receptor name	Natural ligands
α-chemokine (CXC-chemokine)	CXCR1 (IL-8RA)	IL-8, GRO-α/MGSA-α, NAP-2
	CXCR2 (IL-8RB)	IL-8, GRO-α, -β, -γ/ MGSA-α, -β, -γ, NAP-2, ENA-78, IP-10, Mig
	CXCR3	IP-10, Mig
	CXCR4 (fusin, LESTR, humstr, LCR-1)	SDF-1
β-chemokine (CC-chemokine)	CCR1	MIP-1α, -1β, MCP-3, RANTES
	CCR2a (MCP-1RA)	MCP-1, -2, -3
	CCR2b (MCP-1RB)	MCP-3
	CCR3	Eotaxin, RANTES, MCP-2, -3, -4
	CCR4	MIP-1α, RANTES, MCP-1
	CCR5	MIP-1α, -1β, RANTES
	CCR6	MIP-1α, -3α
	CCR7	MIP-3β, Secondary lymphoid tissue chemokines (SLC)

I.4.2. Chemokine receptor gene mutations

Chemokine receptor gene mutations have been described which may affect HIV infection and disease progression and can be divided into three groups.

i) Mutations in protein structure: CCRΔ32, CCR5-m303 and CCR2-64I

A 32 base pair deletion (Δ32) in the CCR5 gene has been described (Paxton *et al*, 1996; Liu *et al*, 1996; Samson *et al*, 1996[a]). Homozygosity for CCR5Δ32 is found in Caucasian populations, in Northern Europe in particular, with an allele frequency of 0.092 but is absent in African and Asian populations (Samson *et al*, 1996[a]). CCR5Δ32 homozygous individuals do not express

CCR5 on the cell surface, preventing viral entry via this co-receptor. Such individuals are apparently resistant to infection with M-tropic viruses. A higher frequency of heterozygosity for CCR5Δ32 has been demonstrated in HIV-1-infected long-term non-progressors (31%) compared with rapid progressors (16%) among homosexual men, but not in haemophiliacs, suggesting that heterozygosity results in delayed disease progression within the homosexual population (Dean *et al*, 1996). CCR5Δ32 heterozygosity in children did not appear to reduce the risk of perinatal acquisition of HIV-1 or delay disease progression (Edelstein *et al*, 1997; Rousseau *et al*, 1997). Although homozygosity for the CCR5Δ32 confers very high resistance to HIV infection, homozygous individuals may still be infected (Biti *et al*, 1997). These individuals might have been infected by an M-tropic strain via a receptor such as CCR1, CCR2b or CCR3 or by a T or dual-tropic virus (Biti *et al*, 1997).

CCR5-m303 is a rarer mutation (frequency of 0.01) with similar effects to Δ32 (Quillent *et al*, 1998; Voevodin *et al*, 1999). The CCR2b-64I mutation is a substitution of valine to isoleucine at amino acid 64 on the CCR2 gene (Smith *et al*, 1997). This mutation is more commonly distributed than CCR5Δ32 and does not affect HIV transmission, but is associated with a two to three year delay in disease progression to AIDS (Kostrikis *et al*, 1998; Rizzardi *et al*, 1998)].

ii) Mutations in regulation of gene expression: e.g. CCR5-59653T

CCR5-59653T is a mutation in the regulatory region of the CCR5 gene (Mummidi *et al*, 1997) and is inherited with the CCR2 mutation, CCR2b-64I (Kostrikis *et al*, 1998). The CCR5-59653T mutation appears to result in reduced expression of CCR5 on the cell surface and therefore may reduce the rate infection of cells by HIV-1 (Kostrikis *et al*, 1998).

iii) Mutation of the chemokine ligand: e.g. SDF-1 3'UTR-801G-A

SDF-1-3'UTR-801G-A is a mutation in the 3' untranslated region of the SDF-1 gene (Winkler *et al*, 1998). The homozygous state appears to result in increased SDF-1 production, which may then compete with X4 strains of HIV-1 for binding to CXCR4 and thus delaying disease progression (Winkler *et al*, 1998).

These mutations appear to have no effect upon the health of the uninfected individual, even in the homozygous state, reflecting the degenerate nature of the large chemokine and chemokine receptor families. Therefore, the lack of adverse effects of these mutations may provide an attractive target for future anti-retroviral therapies.

I.5. TRANSMISSION OF HIV

HIV is transmitted vertically from mother to child *in utero*, during delivery (intrapartum), or postnatally through breast-feeding. HIV-1 is also transmitted by sexual contact, or by contact with infected blood (e.g. blood transfusion, blood products or in intravenous drug users). Although the mechanisms of HIV transmission are not yet fully understood there are certain facts which are well documented including:

i) Transmission of HIV-1 is predominantly restricted to R5 strains (Zhu *et al*, 1993; Roos *et al*, 1992).

ii) HIV-1 isolates from seroconvertors who were infected by sexual contact represent minor variants of the viral species found in the blood of the transmitter (Zhu *et al*, 1996). Sequence homogeneity has also been observed in seroconverting haemophiliacs (Pang *et al*, 1992; Wolfs *et al*, 1992).

I.5.1. Vertical Transmission

Over 90% of children infected with HIV are babies born to HIV^+ women (AIDS epidemic update, December 1999, http://www.unaids.org). Mother-to-child transmission may occur at three different stages: during gestation, during delivery and by breast-feeding (Peckham & Gibb, 1995; Logan *et al*, 1988; Ziegler *et al*, 1985). At least two thirds of infections in non-breastfed children are acquired around the day of birth (during labour and delivery) and

the other one third of infections in non-breastfed children are acquired in the last three weeks of pregnancy (Mock *et al*, 1999; Rouzioux *et al*, 1995; De Rossi *et al*, 1993; Ehrnst *et al*. 1991). Breast-feeding may contribute to between one-third and two-thirds of infections in infants (Simonon *et al*, 1994; Ekpini *et al*, 1997). Encephalopathy, opportunistic infections, and death before 2 years of age are the most severe clinical manifestations of HIV-1 disease in children (Rousseau *et al*, 1997). Reported vertical transmission rates vary from 13% in Europe to 40% in Africa in HIV-1 infected pregnant women receiving no anti-retroviral therapy (Bryson, 1996). Factors associated with increased transmission of HIV from mother to infant include increased levels of virus in blood, the absence of neutralising maternal antibodies against the viral envelope, especially to the V3 loop, a reduced $CD4^+$ cell count, the presence of maternal p24 antigenemia at the time of delivery, the presence of HIV-1 with increased *in vitro* replication in macrophages and prolonged labour and rupture of the membranes (Lathey *et al*, 1999; Kuhn *et al*, 1997[b]; Thea *et al*, 1997; Cao *et al*, 1997; Bryson, 1996; Roques *et al*, 1993; Ades *et al*, 1991; Rossi *et al*, 1989). Among these factors the HIV-1 viral load in the blood and genital secretions of the mother appear to play the most important role in mother-to-child transmission. It has been suggested that *in utero* transmission may be associated with cell-free or cell-associated virus crossing the placenta and increased intrapartum risk may be due to maternal-infant transfusions, disruptions of the placental barrier or direct contact with maternal blood or cervicovaginal secretions (Mock *et al*, 1999). Therefore, elective caesarean-section delivery, before the membrane ruptures,

may reduce the risk of vertical transmission by avoiding direct contact with maternal vaginal secretions and infected blood (European Mode of Delivery Collaboration, 1999). HIV-1 has been detected in oropharyngeal and gastric aspirates of neonates born to HIV-1 infected mothers (Mandelbrot *et al*, 1999). The foetus may be exposed to HIV present in cervicovaginal secretions (Mostad and Kreiss, 1996), through the mucosae, oropharynx and digestive tract (Mandelbrot *et al*, 1999).

Such types of exposure may be a mechanism for postnatal transmission through breast feeding as well as perinatal transmission (Leroy *et al*, 1998; Mandelbrot *et al*, 1999). HIV-1 is present as free-virus (Lewis *et al*, 1998) and cell (macrophage and T cell)-associated virus in milk of HIV-infected mothers (Vonesch *et al*, 1992; Southern, 1998). Mastitis [an inflammatory process in the breast allowing inflammatory cells and extracellular fluid to enter the milk (Thomsen *et al*, 1984)] raises HIV-1 load in breast milk and hence may increase the risk of transmission of HIV-1 from mother to infant (Semba *et al*, 1999).

I.5.2. Transmission via contact with HIV-infected blood

Transmission of HIV by blood transfusions, plasma, or blood cell products (e.g. platelets) occurs either via free virus or virus-infected cells (Ward *et al*, 1989). Studies of transfusion recipients have suggested that the clinical stage of the infected donor (e.g. large number of virus-infected cells, free virus, or

the type of virus present in the donor's blood) can influence the progression to disease in the recipient.

I.5.3. Sexual Transmission

Heterosexual transmission is responsible for more than 90% of HIV-1 infections in Africa and is increasing more rapidly than all other modes of transmission in the US, Europe, and most other regions of the world (Caceres and Hearse, 1996; Mastro and De Vincenzi, 1996; Lee *et al*, 1996; http://www.unaids.org). The rate of heterosexual transmission has been estimated to be approximately one in every 1000 coital acts in both North American (Padian *et al*, 1997) and Uganda (Gray *et al*, 2001) couples where one partner was HIV-1 positive. In these studies, higher rates of transmission were highly associated with genital ulceration and higher plasma viral loads.

In order to establish infection within the female via the heterosexual route of transmission, HIV must cross the epithelial barrier of the female reproductive tract and ultimately infect $CD4^+$ T cells and macrophages either within the underlying submucosal tissue or within the draining lymph nodes, with subsequent systemic infection. Therefore, the transfer of HIV across the mucosal epithelium is a key event in sexual transmission of HIV-1.

The surface of the female genital tract is covered with epithelial cells which provide a protective barrier for the underlying tissue and is also covered with

antimicrobial secretions including lactoferrin, lysozyme and both transmembrane mucins (eg. Mucin 1 or episialin) and secretory mucins (DeSouza *et al*, 1999; Gendler and Spicer, 1995; Hilkens *et al*, 1992; Lamblin and Roussel, 1993). The epithelial cells in the female genital tract are composed of columnar, squamous and transitional cells. The vagina and ectocervix, the tissues which would be exposed to HIV^+ semen to the greatest extent, are comprised of five distinct cell layers: from the lumen the first three layers are the superficial, transitional, and intermediate layers (fig.I.4). Each is composed of approximately 10 rows of squamous epithelial cells ('stratified epithelium'). Beneath these layers are the parabasal and basal layers, containing one to two rows of columnar epithelial cells (Witkin, 1993). In contrast, the endocervix and uterus are composed of a single layer of epithelial cells ('simple epithelium') which is similar to the rectum. This may explain the higher frequency of rectal transmission in homosexual men, along with the greater likelihood of epithelial damage during anal sex. HIV-1 susceptible cells, Langerhans' cells, $CD4^+$ T cells and macrophages are predominantly present in the epithelium, parabasal and basal layers of the vagina and ectocervix (Johansson *et al*, 1999; Poppe *et al*, 1998; Morris *et al*, 1983).

Several mechanisms have been proposed to mediate and/or facilitate the transfer of HIV-1 across the mucosal epithelium:

i) <u>Direct infection of epithelial cells with HIV</u>

Fig.I.4. The female genital tract

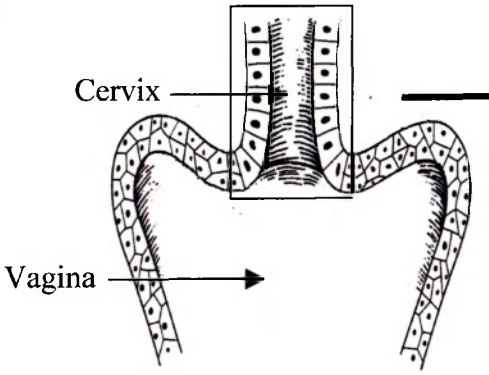
(a) Section through the vagina and cervix (adapted from Hall-Craggs, 1990).

(b) Schematic diagram of cervix showing the endocervix and ectocervix.

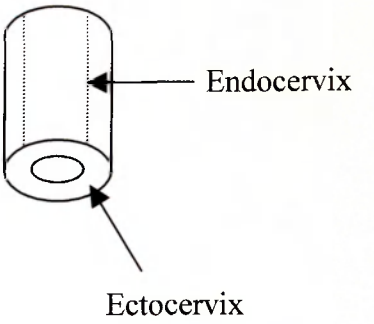
(c) Typical cornified original squamous epithelium of the vagina and ectocervix showing five well-defined layers: from the lumen, superficial (1), transitional or intraepithelial (2), intermediate (3), parabasal (4), and basal (5) layers (adapted from Jordan & Singer, 1976).

(d) Normal columnar lining epithelium of the endocervix (adapted from Patten, 1978). The endocervix is composed of a single layer of columnar epithelial cells. Most lymphocytes ($CD4^+$ T cells, $CD8^+$ T cells and macrophages) reside within the lamina propria in close proximity to the basal membrane. In addition, the majority of Langerhans cells also reside within close proximity to the basal membrane but may be found in both the epithelium and lamina propria layers.

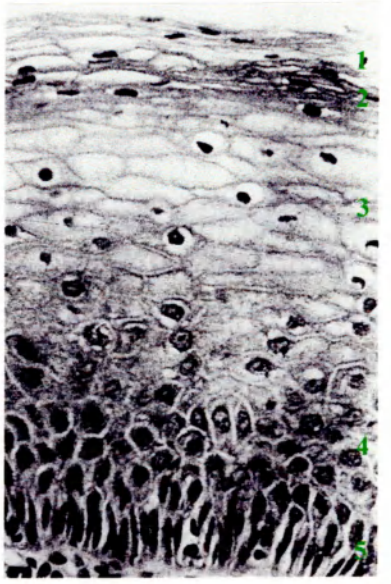
(a)



(b)



(c)



(d)

HIV-1 has been shown to infect the human cervical epithelial cell line ME180 *in vitro* (Tan *et al*, 1993). Exposure of this cell line to cell-free HIV-1 resulted in non-productive infection demonstrated by DNA PCR. However, productive and cytopathic infection followed exposure of ME180 cells to HIV-infected T cell lines. HIV-1 has also been shown to transcytose through tight monolayers of human intestinal and endometrial epithelial cell lines *in vitro* (Bomsel, 1997). Therefore, these studies describe a possible efficient mechanism for heterosexual transmission of HIV-1, with virus crossing the intact genital epithelial barrier and gaining access to the submucosal $CD4^+$ cells. However, the relatively low rate HIV-1 transmission per heterosexual coital act described above would argue against such an efficient mechanism. Other studies employing primary cultures of human epithelial cells have demonstrated the limitations of studies using transformed cell lines, which may bear little relation to the *in vivo* situation. HIV-1 infection has been demonstrated in cell preparations derived from the human cervix, but the infection was restricted to $CD45^+$ leukocytes with no evidence of epithelial cell infection (Patrick *et al*, 1993). HIV-1 virions have been shown to associate with human primary cervical epithelial cells and remain in an extracellular location (Dezzutti *et al*, 2001) with virus then being transferred to activated PBMCs. However, these cultures were not polarised monolayers of cells and therefore would not equate to the intact epithelium found *in vivo*. Polarised cervical epithelial cultures have been shown to be an effective barrier against HIV-1 (Greenhead *et al*, 2000). Neither productive infection nor virion transcytosis could be detected following exposure of intact cervical

epithelium to both cell-free and cell-associated HIV-1. Non-polarised cultures were productively infected with HIV-1, with the target cells residing within the submucosal tissue and infection enhanced by immune activation.

ii) Disruption of the epithelial barrier

For many sexually transmitted diseases (STDs) including HIV infection, mucosal integrity may be an important factor affecting transmission. Transmission of HIV would be particularly likely if there are open lesions or ulcers and inflammatory cells present due to other STDs, thereby providing a portal for HIV entry (Wasserheit, 1992; Dickerson *et al*, 1996; Plummer, 1998; Cohen, 1998; Gray *et al*, 1999). The one in 1000 rate of heterosexual transmission described previously (Padian *et al*, 1997; Gray *et al*, 2001) is increased approximately four-fold in the presence of ulcerative genital disease (Gray *et al*, 2001). In HIV^+ patients, genital tract shedding of HIV is increased with ulcerative STDs (Ghys *et al*, 1997; Schacker *et al*, 1998; Dyer *et al*, 1998; Gadkari *et al*, 1998) and treatment reduces but does not abolish HIV shedding (Gadkari *et al*, 1998; Ghys *et al*, 1997). Bacterial vaginosis may also increase susceptibility to HIV-1 infection through loss of naturally occurring virucidal mechanisms. Bacterial vaginosis results in an absence of hydrogen peroxide-producing lactobacilli (Klebanoff and Coombs, 1991) and a reduction in myeloperoxidase activity (Klebanoff and Coombs, 1992). Furthermore bacterial pathogens such as *C. trachomatis* both recruit and interact with polymorphonuclear leukocytes (PMN) *in vivo* and the activated PMN in turn activate mononuclear cells by generating reactive oxygen

intermediates during phagocytosis of the bacteria and hence increase HIV replication (Ho *et al*, 1995). In summary, the potential mechanisms by which STDs may increase HIV transmission include:

1) Increased number or activation of macrophages, $CD4^+$ T cells or DCs in the genital tract (Levine *et al*, 1998; Greenhead *et al*, 2000).
2) Disruption of the epithelial barrier (Kiviat *et al*, 1990).
3) Reduction in T-helper and T-cytotoxic function (Mazzoli *et al*, 1997).
4) Shedding of inflammatory mediators in semen (Ramsey *et al*, 1995) which may activate DCs, macrophages and T cells in the mucosa of the female reproductive tract.

Contraception also influences transmission of HIV. Condoms are lubricated with a spermicide containing nonoxynol-9. In addition spermicidal sponges for use by the female also contain nonoxynol-9. Nonoxynol-9 is active against HIV and other sexually transmitted pathogens (Stafford *et al*, 1998) by disruption of cell membranes and viral envelopes (Reitmeijer, 1988). However, nonoxynol-9 may also damage the epithelium of the female reproductive tract, thereby increasing susceptibility to HIV infection. Hormonal contraception has been shown to increase susceptibility to HIV infection (Plummer *et al*, 1991). This may be due to cervical ectopy or thinning of the vaginal epithelium, in progesterone only contraception in particular (Marx *et al*, 1996).

iii) Infection or association of HIV with epithelial Langerhans' cells

Mucosal epithelial dendritic cells (DCs) [Langerhans' cells (LC)] have been suggested to be the first cells to be infected after mucosal exposure to HIV as shown by studies using rhesus macaque models of Simian immunodeficiency virus (SIV) infection (Zambruno *et al*, 1995; Spira *et al*, 1996; Blauvelt, 1997; Joag *et al*, 1997; Miller and Hu 1999) and using skin explants (Reece *et al*, 1998). DCs may then transfer HIV to T cells within the lymph node followed by systemic spread of infection (Spira *et al*, 1996; Masurier *et al*, 1998). As this possibility is a focus of this thesis, DC biology and association with HIV is now discussed.

I.6. The Biology of Dendritic cell

The literal meaning of a dendritic cell would be a cell that possesses dendrites. This term could therefore be applied to many cell types including brain and nerve cells and macrophages. However, in the field of immunology, the term dendritic cell (DC) is used for a population of cells commonly termed antigen presenting cells (APC). These DCs have the unique ability to take up, process and present antigen in association with MHC class II to CD4 T cells followed by stimulation of naive T cells, thereby initiating a primary immune response to an antigen. However, DCs, and macrophages, may also present antigen to and stimulate memory T cells. The biology of this dendritic cell has been recently reviewed by Hart (1997) and Banchereau *et al* (2000).

Another cell with immunological function with dendritic morphology is the follicular dendritic cell (FDC) which is found in the germinal centres of the lymph node. FDCs are derived from a separate lineage to DCs. FDCs have the capacity to capture and retain whole antigens in the form of immune complexes and play a critical role in the generation of the humoral immune response and also in activation and selection of B cells (Grouard and Clark, 1997). B cells recognise whole antigen via cell surface expression of antibody. Unless stated, all references to DCs in this thesis relate to the T cell stimulatory lineage of dendritic cells rather than the FDC.

DC functioning from antigen capture to T cell stimulation involves several individual steps requiring alteration of the DC functional abilities and phenotype, with these steps occurring in separate microenvironments within the body, requiring migration of the DC. The following is a summary of the DC life cycle and function:

i) DCs are $CD45^+$ bone marrow derived leukocytes. Immature DCs migrate to non-lymphoid tissues via the blood stream where they may encounter antigen. One well characterised tissue DC is the Langerhans cell (LC) located in the epithelium of the skin and mucosa. Mucosal tissues in particular would be expected to contain numerous DCs in order to meet the greater antigenic challenge at these exposed sites. In addition, inflammation leads to rapid accumulation of DCs in infected or damaged tissues.

ii) Immature DCs in the tissues capture and internalise antigen efficiently via several pathways including macropinocytosis, receptor mediated endocytosis and phagocytosis.

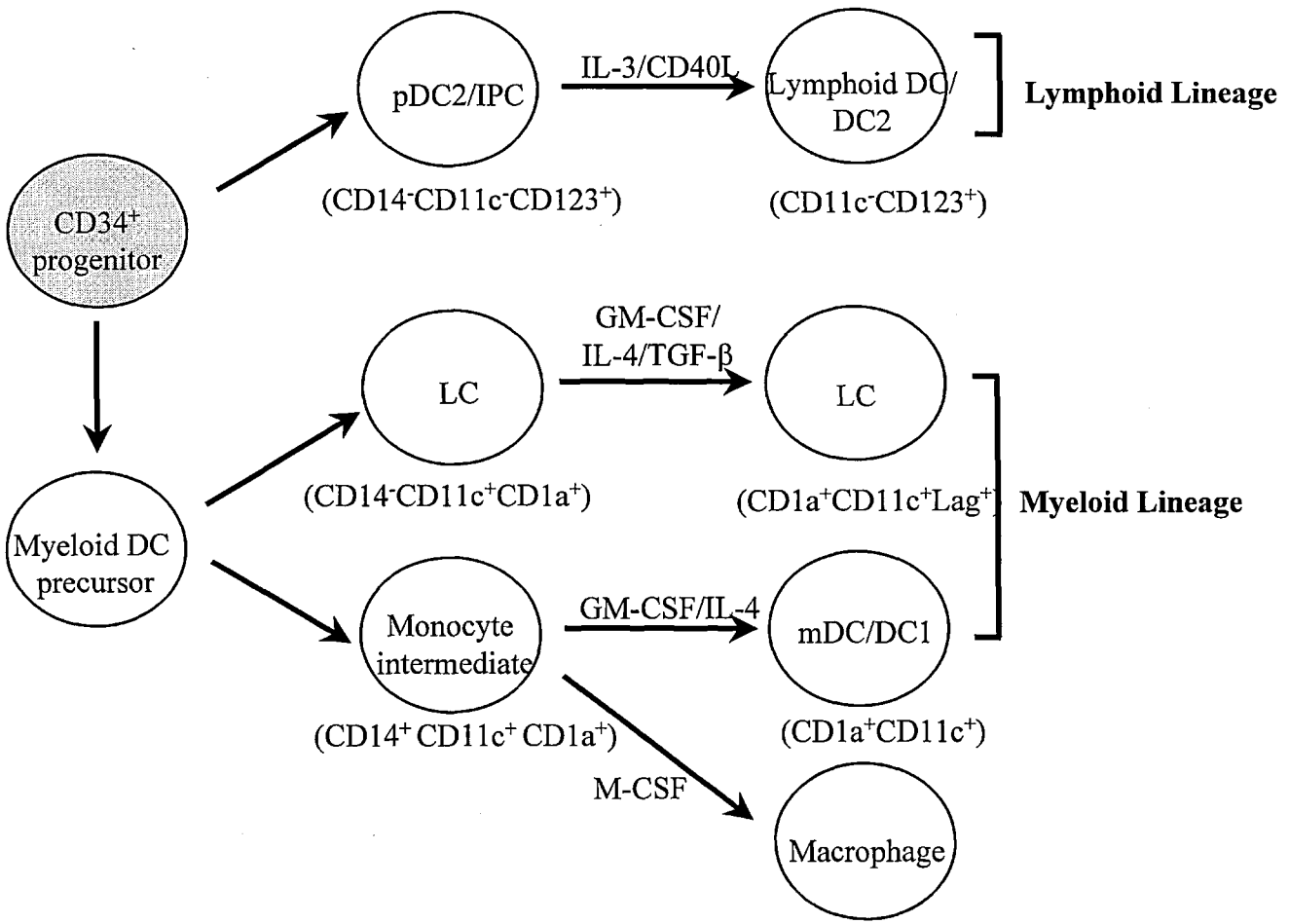
iii) Maturation of the DC to an antigen presenting phenotype is associated with migration of the DC from the tissue to the lymph node via the afferent lymphatics. Therefore, antigen sampled in the tissues is transported to lymphocyte rich lymphoid tissue. Maturation involves a reduction in phagocytic capacity, up-regulation of T cell co-stimulatory molecules and presentation of processed antigen in association with MHC molecules.

iv) Prior to antigen presentation and T cell stimulation, the migrated DC may associate with several T cells in the lymph node via interaction between adhesion molecules, forming a DC-T cell cluster. This process would facilitate recognition of the presented antigen by a specific T cell.

v) Following recognition by an antigen-specific T cell, the DC stimulates the naïve T cell via expression of co-stimulatory molecules such as CD80 and CD86, thereby inducing a primary immune response to the antigen. In addition the DC may receive stimulatory signals from the T cell via interaction between CD40 expressed on DCs and CD40 ligand (CD40L/CD154) expressed on T cells.

There are at least three distinct subpopulations of DCs (fig. I.5): two in the myeloid lineage [LCs and interstitial (dermal) DCs/DC1] and one in the lymphoid lineage (lymphoid DCs). $CD123^{+}CD11c^{-}$ pre-DCs (pDC2), also known as plasmacytoid or IFN-α producing cells [IPC (Cella *et al*, 1999;

Fig.I.5. Developmental pathway of DCs



Siegal *et al*, 1999)], give rise to lymphoid DCs [$CD11c^{-}CD123^{+}$ (also known as DC2)] when cultured and activated with IL-3 and CD40 ligand (CD40L) (Grouard *et al*, 1997; Olweus *et al*, 1997). CD40L-activated DC2 produce IL-1α, IL-1β, IL-6, IL-8 and IL-10 but no IL-4 (Rissoan *et al*, 1999) and may preferentially stimulate Th2-type responses (Rissoan *et al*, 1999; Pulendran *et al*, 1999). The myeloid lineage DCs can be further differentiated into two distinct subsets. One is the LC precursor cell-derived LC subset ($CD14^{-}$ $CD11c^{+}CD1a^{+}$) and the other is the monocyte-derived DC subset ($CD14^{+}CD11c^{+}CD1a^{+}$) which is also known as interstitial (dermal) DCs (Nestle *et al*, 1994) or DC1 (Rissoan *et al*, 1999). Monocyte-derived DCs produce large amounts of IL-12 on CD40L activation and may preferentially stimulate Th1-type response (Rissoan *et al*, 1999; Pulendran *et al*, 1999). The terms DC1 and DC2 are based on the cytokine profile produced by these cells, those which produce type 1 cytokines are called DC1 and those which produce type 2 cytokines are called DC2. The corresponding mature DC progeny of both subsets of myeloid lineage DCs are equally potent in stimulating the proliferation of naïve T cells, but only interstitial DCs induce the IL-2-driven differentiation of naïve B cells *in vitro* (Caux *et al*, 1997).

Immature DCs in non-lymphoid tissues are specialised in the uptake and processing of antigens. DCs employ three distinct mechanisms for antigen uptake:

i) Fluid phase antigen uptake by macropinocytosis which is constitutive and allows continuous internalisation of large volumes of fluid (Sallusto *et al*, 1995).

ii) Receptor-mediated endocytosis via C-type lectin receptors such as the mannose receptor (Engering *et al*, 1997; Cella *et al*, 1997; Jiang *et al*, 1995; Sallusto *et al*, 1995), DC-SIGN (Geijtenbeek *et al*, 2000[a]) and DEC-205 which is expressed on murine DCs and thymic epithelial cells (Jiang *et al*, 1995)] or via Fc receptors [e.g. FcγRI (CD64), FcγRII (CD32), FcεRI and low-affinity FcεRII (CD23) (Cella *et al*, 1997; Fanger *et al*, 1996]. Mannose receptors bind to carbohydrate antigens, mediate endocytosis and release antigen in to a multivesicular endosome and then recycles back to the cell-surface, allowing continuous internalisation of antigen and maintaining a capacity for antigen capture (Cella *et al*, 1997; Sallusto *et al*, 1995). Human LCs lack functional mannose receptors and have poor endocytic capacity (Mommaas *et al*, 1999). However LCs do express an LC-specific C-type lectin (Langerin) (Valladeau *et al*, 1999[a]; Valladeau *et al*, 1999[b]).

iii) Phagocytosis of particles [e.g. apoptotic and necrotic cell fragments (Albert *et al*, 1998[a]; Albert *et al*, 1998[b]), bacteria (Inaba *et al*, 1993) and intracellular parasites (Moll *et al*, 1993)].

It is known that processed exogenous antigens in the form of short peptides are presented to CD4 T helper cells in a MHC class II-restricted manner. There is evidence for MHC class I-restricted presentation of exogenous antigens taken up by DCs by macropinocytosis or in a receptor-mediated

manner and are then presented to $CD8^+$ CTLs (Rock *et al*, 1993; Norbury *et al*, 1995; Jondal *et al*, 1996; Norbury *et al*, 1997; Mitchell *et al*, 1998; Regnault *et al*, 1999). Several groups have attempted to define the mechanisms for MHC class I-restricted exogenous antigen presentation. Normal and TAP mutant cells and cell lines have been used along with cellular inhibitors selective for Golgi transport (Brefeldin A) or proteasomal activity (lactacystin and peptide aldehydes) and inhibitors targeting endosomal functions such as vesicular pH, proteolysis and transport. The suggested mechanisms for exogenous antigen presentation in a MHC class I-restricted manner are as follows:

i) Brefeldin A-sensitive conventional pathway.

Internalised antigens may be released into the cytosol from the endocytic compartment for classical TAP-dependent MHC class I presentation (Norbury *et al*, 1997; Kovacsovics-Bankowski and Rock, 1995). The mechanism of antigen release into the cytosol is not fully understood but there is evidence for the involvement of carrier molecules or chaperones (Schirmbeck and Reimann, 1994; Jondal *et al*, 1996).

ii) Brefeldin A-insensitive pathway

Internalised antigens may be processed in endocytic compartments, generating peptides which are loaded onto post-Golgi MHC class I molecules in endosomes or at the cell surface after peptide regurgitation. (Liu *et al*, 1995; Schirmbeck *et al*, 1995; Harding and Song, 1994; Jondal *et al*, 1996).

Trafficking of DCs is regulated by cytokines and chemokines. Pro-inflammatory cytokines including IL-1 and TNF-α promote LC emigration (Cumberbatch *et al*, 1990; Wang *et al*, 1999). LC precursors ($CD1a^+CD14^-$) express CCR6 and respond selectively to MIP-3α secreted by keratinocytes (Charbonnier *et al*, 1999). $CD14^+$ DC precursors ($CD1a^-CD14^+$) which differentiate into macrophage or monocyte-derived DCs (MDDCs) and are more closely related to dermal DCs or interstitial DCs, respond to MIP-1α, MCP-3 and RANTES which can mediate the migration of these cells to injured or inflamed peripheral tissues (Charbonnier *et al*, 1999). Immature DCs express CCR1, CCR2, CCR3, CCR5 and CXCR1 (Rubbert *et al*, 1998; Sallusto *et al*, 1998; Yanagihara *et al*, 1998; Dieu *et al*, 1998). Maturation of DCs up-regulates expression of receptors for chemokines produced in lymphoid organs and down-regulates expression of receptors for inflammatory cytokines (Cyster, 1999; Sallusto *et al*, 1998; Sozzani *et al*, 1998[a]; Yanagihara *et al*, 1998; Sallusto *et al*, 1999; Sallusto and Lanzavecchia, 1999). Mature DCs express high levels of CCR7, acquiring responsiveness to the Secondary Lymphoid tissue Chemokine (SLC / 6Ckine) and to EBV-induced molecule 1 Ligand Chemokine (ELC) [also known as MIP-3β (Yoshida *et al*, 1997)] (Sallusto *et al*, 1998; Yanagihara *et al*, 1998; Dieu *et al*, 1998; Cyster, 1999). Maturation also results in up-regulation of CXCR4 (Canque *et al*, 1999; Sallusto *et al*, 1998; Zaitseva *et al*, 1997), whose natural ligand, SDF-1 is constitutively secreted in lymphoid tissues (Delgado *et al*, 1998; Sallusto *et al*, 1998; Austyn 1998). Mature DCs display

reduced expression of CXCR1, CCR1, CCR5 (Sallusto *et al*, 1998) and CCR6 (Dieu *et al*, 1998; Carramolino *et al*, 1999).

For DC and T cell interactions to occur in lymph node T cell areas, adhesion molecules (Tsunetsugu-Yokota *et al*, 1997; Prickett *et al*, 1992; Starling *et al*, 1995; King *et al*, 1989), cytokines/chemokines (Sallusto *et al*, 1998; Adema *et al*, 1997) and co-stimulatory molecules (Gribben *et al*, 1995; Pinchuk *et al*, 1994; Caux *et al*, 1994) are required. DC-T cell cluster formation requires an intact DC cytoskeleton and protein kinase C activation to initiate the adhesive interaction (Prickett *et al*, 1990; Scheeren *et al*, 1991). Examples of adhesion molecules involved in DC-T lymphocyte interaction include (fig.I.6):

i) LFA-1 on DCs and ICAM-3 on resting T cells (Starling *et al*, 1995; Hauss *et al*, 1995).

ii) DC-SIGN on DCs and ICAM-3 on T cells (Geitenbeek *et al*, 2000[a]).

iv) LFA-3 on DCs and CD2 on T cells (Prickett *et al*, 1992; King *et al*, 1989).

DCs in the lymph node T cell areas secrete cytokines that act as chemoattractants for T cells. For instance, ELC/MIP-3β secreted by mature DCs is involved in attracting both T cells expressing CCR7 as well as antigen carrying DCs (Sallusto *et al*, 1998), DC-CK1 selectively attracts naïve T cells (Adema *et al*, 1997). SDF-1, MCP-1, RANTES, MIP-1α and MIP-1β are also secreted by mature DCs (Sallusto *et al*, 1998). Expression of co-stimulatory molecules on DCs is enhanced on activation. Among the most important co-

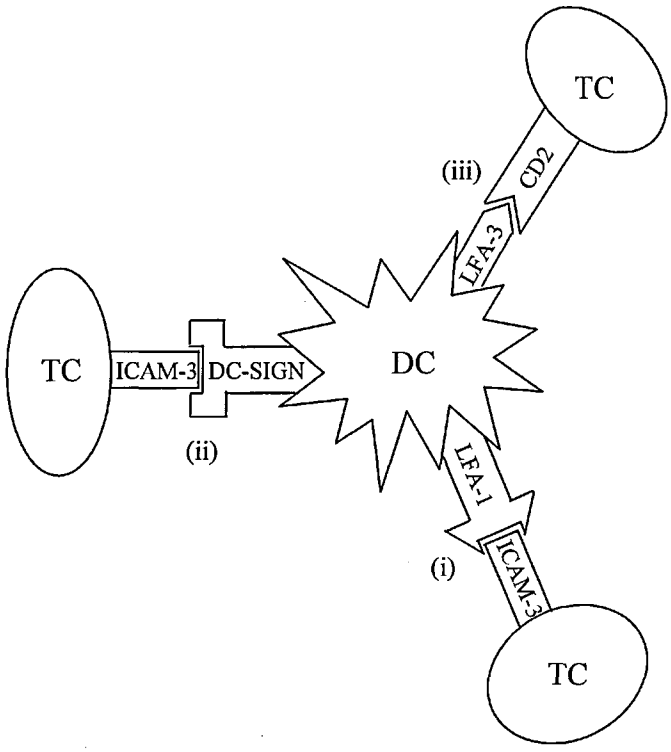


Fig.I.6. Examples of adhesion molecules involved in dendritic cell (DC) and T cell (TC) interaction

Intracellular adhesion molecule (ICAM)-3 on resting TC binds to (i) leukocyte function antigen (LFA)-1 or (ii) DC-SIGN expressed on DC. CD2 on TC binds to LFA-3 on DC (iii).

stimulatory signals are those delivered by members of the B7 family, which is to date composed of six members. B7.1 (CD80) and B7.2 (CD86), which bind to different sites of the CD28 molecule or to CTLA-4 on T cells, are capable of delivering a positive co-stimulatory signal (Thompson *et al*, 1989; Linsley *et al*, 1991[a]) or a counter-regulatory negative signal (Linsley *et al*, 1991[b]; Waterhouse *et al*, 1996), respectively. B7-H1 (PD-L1) and B7-H2 (B7-RP1) bind to the PD-1 (Freeman *et al*, 2000) and inducible co-stimulator [ICOS (Wang *et al*, 2000; Yoshinaga *et al*, 1999), respectively. The functions of B7-H1 and B7-H2 are not yet well documented. B7-H3 binds to a putative receptor on activated T cells that is distinct from CD28, CTLA-4, PD-1 and ICOS (Chapoval *et al*, 2001). B7-H3 co-stimulates proliferation of both $CD4^+$ and $CD8^+$ T cells, enhances the induction of cytotoxic T cells and selectively stimulates IFN-γ production by T cells (Chapoval *et al*, 2001). Expression of five members of B7 family listed above is not restricted to DCs, but B7-DC is expressed exclusively, as far as is known, on DCs and monocyte-derived DCs (Tseng *et al*, 2001). B7-DC which binds to PD-1, was shown to co-stimulate T cell proliferation and to induce IFN-γ but not IL-4 or IL-10 production by naïve T cells in mice (Tseng *et al*, 2001). CD40, a member of the TNF receptor family, is expressed on DCs and interacts with CD40L (CD154) (McLellan *et al*, 1996) expressed on activated, but not resting T cells (Caux *et al*, 1994). This induces IL-12 production by the DC (Shu *et al*, 1995), which is in turn required for activation of Th1 T cells.

I.6.1 Dendritic cells and their potential role in HIV-1 transmission

DC susceptibility to HIV infection has been shown both *in vivo* and *in vitro* (Patterson *et al*, 1994; Patterson *et al*, 1995), although the proviral load in T cells is 3-100 times higher than that in DCs (Patterson *et al*, 1998). There is evidence for transfer of virus between DCs and T cells by phylogenetic analysis of the V3 loop and flanking regions of the virus isolated from symptomatic HIV patients' DCs and T cells (Patterson *et al*, 1998). *In vitro* studies showed that HIV infection of DCs can be blocked by neutralising monoclonal antibodies and hence cell-to-cell transmission of the virus from DCs to T cells can be halted (Frankel *et al*, 1998). In addition, immature DCs can transmit R5 viral strains to blood monocytes and monocyte-derived macrophages with higher efficacy than mature DCs, with the CD11/CD18 family of adhesion molecules mediating DC-monocyte/macrophage interaction (Kacani *et al*, 1998).

There is evidence that subepithelial DCs, may be the first cells to be infected after mucosal exposure to HIV, as has been shown in studies using rhesus macaque models of SIV (Spira *et al*, 1996; Joag *et al*, 1997; Miller and Hu, 1999) and using skin explants (Reece *et al*, 1998). Therefore, it has been suggested that LCs may play a role in establishing HIV infection in lymph node T cells (Spira *et al*, 1996; Masurier *et al*, 1998). However, it has also been suggested that endocervical T cells may be the first cells to be infected in the female (Zhang *et al*, 1999) and this is discussed in chapter VI. DCs express CD4 and the co-receptors required for HIV fusion and entry

(Patterson *et al*, 1995; Wright-Browne *et al*, 1997; Lee *et al*, 1999[a]). The newly identified DC-specific ICAM-3 receptor, DC-SIGN, was also shown to bind to gp120 and hence to be involved in capture of HIV-1. Furthermore, an interaction between DC-SIGN and ICAM-3 facilitates the infection of T cells (Geijtenbeek *et al*, 2000[b]). Freshly isolated LCs from skin epidermis express CCR5, the principal co-receptor for fusion and entry of R5 viral strains. These LCs do not express CXCR4 (Zaitseva *et al*, 1997) providing an explanation for the preferential sexual transmission of R5 viral strains. Such data does not however explain the M-tropic-restricted transmission that also occurs via other routes including vertical transmission and blood. M-tropic strains of virus, but not T-tropic strains, may enter DCs derived from the tonsil and thymus (Cameron *et al*, 1996). Peripheral blood DCs which are generally thought to be immature DCs, express CCR5 but low levels of CXCR4 (Lee *et al*, 1999[b]). Expression of CXCR4 may be increased on DC maturation (Zaitseva *et al*, 1997; Zoeteweij *et al*, 1998; Lee *et al*, 1999[b]). Expression of CXCR4 is also regulated by cytokines as is demonstrated by the fact that Th2 type cytokines, IL-4 and TGF-β1 up-regulate, whereas Th1 type cytokines IFN-α, IFN-β and IFN-γ inhibit CXCR4 expression (Zoeiteweil *et al*, 1998). A relative increase in type-2 cytokines, which can occur during HIV infection (Stylianou *et al*, 1999; Meroni *et al*, 1996), may up-regulate CXCR4 expression on mature DCs favouring infection with X4 viral strains (Zoeteweij *et al*, 1998). HIV infection of DCs is blocked by the natural co-receptor ligands RANTES and SDF-1 (Rubbert *et al*, 1998; Granelli-Piperno *et al*, 1996). Evidence for the presence of a SDF-1 receptor other than

CXCR4 on DCs, which is used by both R5 and X4 viral strains, has been shown by a study of HIV infected individuals homozygous for Δ32CCR5 and by an inhibition assay with SDF-1 (Rubbert *et al*, 1998).

I.7. SEMEN

I.7.1. Composition of seminal fluid

Seminal fluid consists of various secretions of the male accessory reproductive organs (Geigy Scientific Tables, vol.1, 8th edition)(fig.I.7). The three main secretions are acid phosphatase (prostatic secretion), spermatozoa (testicular and epididymal secretion) and fructose (seminal vesicle secretion). About 10% of the dry mass of the total ejaculate consists of inorganic materials and 90% is organic (Table I.3). Some semen specimens contain antibodies of the IgA type which are capable of agglutinating spermatozoa and are significant with respect to male infertility. The normal pH of semen is slightly alkaline (7.2-8.0). Cell types other than spermatozoa in human semen include leukocytes, epithelial cells and immature germ cells. These are collectively referred to as 'non-spermatozoal cells' (NSC) or 'round cells'. Leukocytospermia (leukocyte counts in seminal fluid of more than $2x10^6$/ml) may indicate infection in the reproductive tract and large numbers of epithelial cells in semen may be an indication of infection or overactive masturbation. The presence of erythrocytes may indicate reproductive tract

Table I.3. Substances found in seminal fluid.

Material	Example	Characteristics
Inorganic substances	Chloride	-
	Phosphorus	As phosphorylcholine and glycerylphosphrylcholine
	Potassium	Mostly from the prostatic secretion; concentration increased in cystic fibrosis
	Sodium	-
	Calcium	Mostly from the prostatic secretion; concentration increased in cystic fibrosis
	Magnesium	Mostly from the prostatic secretion; concentration increased in cystic fibrosis
	Zinc	Almost exclusively from the prostatic secretion; bound to glycoproteins; concentration increased in cystic fibrosis and decreased in prostatitis
	Copper	-
Nitrogenous substances	Ammonia	Increased on prolonged incubation at 37°C due to progressive proteolysis
	Amino acids	24 different amino acids identified
	Choline	Released from phosphorylcholine by acid phosphatase
	Phosphrylcholine	Hydrolysed to form phosphate and choline within 1 hour of ejaculation
	Glycerylphophoryl-choline	From the epididymis
	Carnitine	Mainly from the epididymis
	Polyamines	Putrescine, Spermidine, Spermine (refer text); from the prostatic secretion
	Proteins	Gradually broken down by proteolytic enzymes and peptide hydrolases; eg. albumin, transferrin, lactoferrin, IgA, IgG, IgM, Lysozyme, Lactate dehydrogenase and acid phosphatase
Carbohydrate & Metabolites	Glucose	Energy source for the spermatozoa
	Fructose	Formed almost entirely in the seminal vesicles; most important energy source for the spermatozoa
	Sialic acid	Equally from both prostatic secretion and seminal vesicles
	Fucose	-
	Myoinositol	Mostly from the prostatic secretion
	Sorbitol	-
	Lactic acid & Pyruvic acid	Metabolites produced by the anaerobic metabolism of the spermatozoa
	Citric acid	Almost exclusively from the prostate
Lipids	Phopholipids	Phosphatidylcholine is preponderant in the plasma
	Cholesterol	-
	Prostaglandin	Refer text
Vitamins	Vit. B_{12} & Ascorbic acid	-
Hormones	Pregnenolone, Androstenedione, Dihydrotestosterone, Estradiol, Prolactin, Follicle-stimulating hormone, etc.	-

Fig. I. 7. The male reproductive system (adopted from Burkitt *et al*, 1993). Section through the male pelvis (a) and testis (b)

The male reproductive system mat be divided into four functional components:

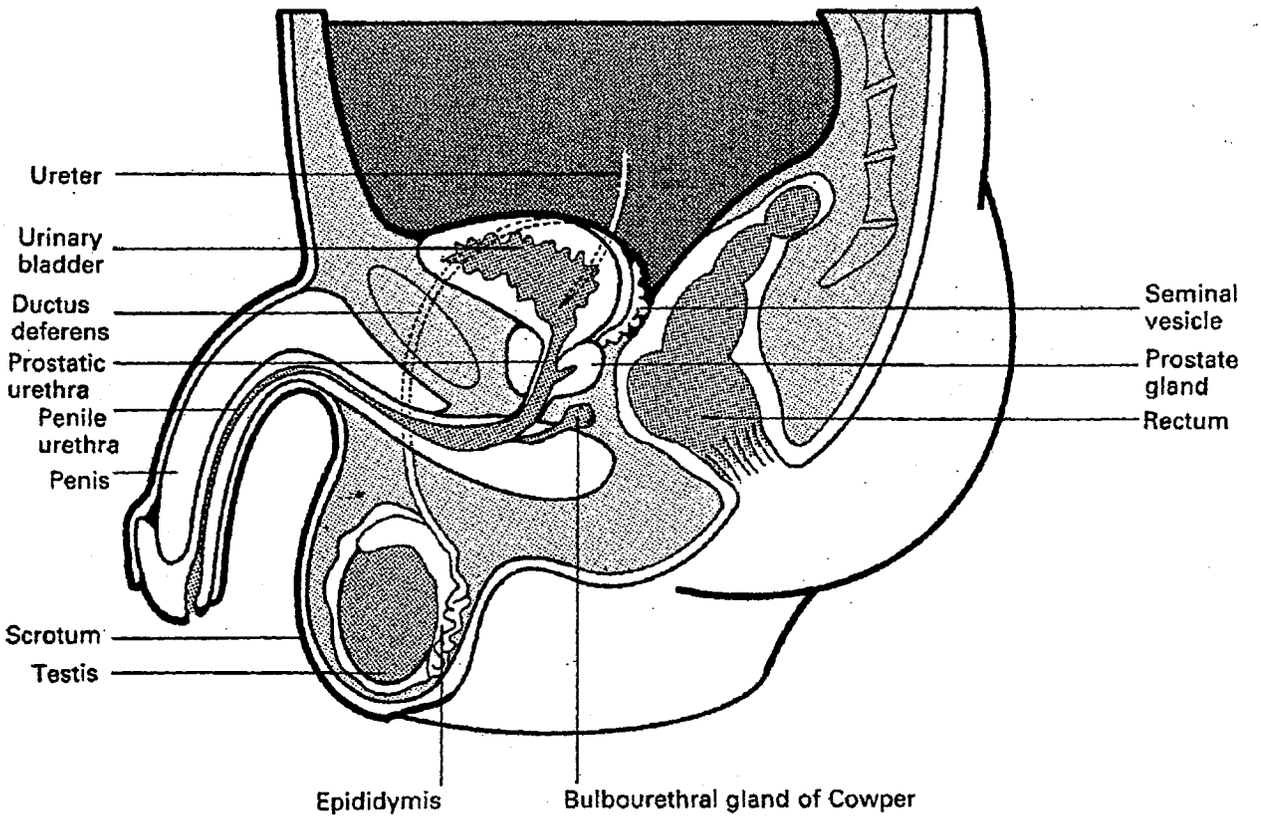
• The testes, paired organs lying in the scrotal sac, are responsible for production of the spermatozoa and secretion of male sex hormones.

• A paired system of ducts, each consisting of ductuli efferentes, epididymis, ductus deferens and ejaculateatory duct, collect, store and conduct spermatozoa from each testis. The ejaculatory ducts converge on the urethra from which spermatozoa are expelled.

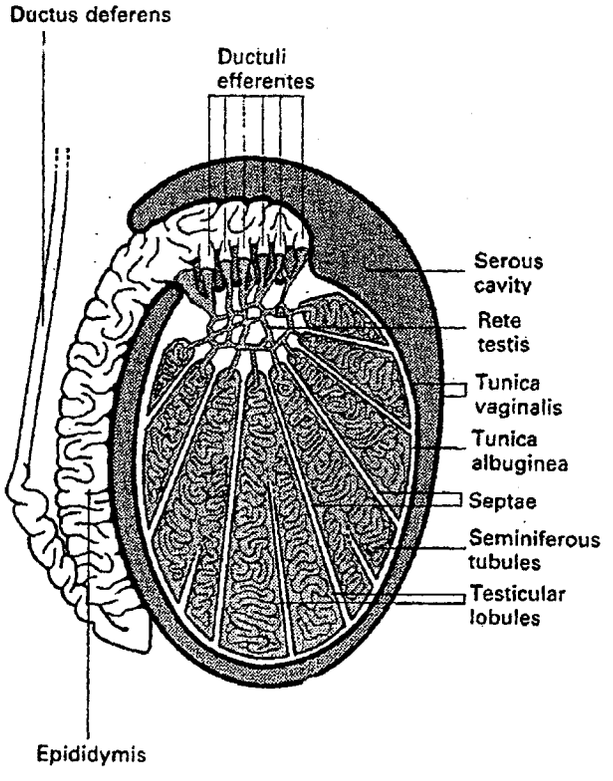
• Two exocrine glands, the paired seminal vesicles and the single prostate gland, secrete a nutritive and lubricating fluid medium (seminal fluid).

• The penis is the organ of copulation. A pair of small accessory glands, the bulbo-urethral glands of Cowper, secrete a fluid which lubricates the urethra for the passage of semen during ejaculation.

(a)


Ureter
Urinary bladder
Ductus deferens
Prostatic urethra
Penile urethra
Penis
Scrotum
Testis
Epididymis
Bulbourethral gland of Cowper
Seminal vesicle
Prostate gland
Rectum

(b)


Ductus deferens
Ductuli efferentes
Serous cavity
Rete testis
Tunica vaginalis
Tunica albuginea
Septae
Seminiferous tubules
Testicular lobules
Epididymis

infection or damage to a small capillary. Ejaculation is immediately followed by coagulation of seminal fluid mediated by a clotting enzyme (proteinase) from the prostate acting on a fibrinogen-like protein from the seminal vesicles. Within 5 to10 minutes of ejaculation, the gelatinous-viscous seminal fluid becomes liquefied due to proteolytic degradation of the coagulation product by a plasmin-like enzyme from the prostate gland. Chymotrysin-like enzymes, dipeptidases and aminopeptidases also hydrolyse the clot fragments to tripeptides, dipeptides and free amino acids. The density and viscosity of the total ejaculate depends on the quantity of spermatozoa. The spermatozoa formed in the testicles migrate into the epididymis, where they are stored and subjected to a maturation process. The number of spermatozoa varies between individuals from less than $2x10^7$/ml to over $8x10^7$/ml. A high proportion of motile spermatozoa benefit fertility. *In vitro*, motility is temperature-dependent and is considerably higher at 37°C than at room temperature. The proportion of highly motile spermatozoa decreases with age. The spermatozoon consists of three parts: a head, mid-piece and tail. The head of spermatozoa contains the nucleus and acrosome. The acrosome is a modified lysozome containing a characteristic lipoglycoprotein complex which is associated with a number of enzymes. Some of the enzymes, in particular a trypsin-like proteinase (acrosin), are important for fertilisation and are involved in breaking open of the ovarian cumulus and penetration of the pellucid zone. The mid-piece of spermatozoa is rich in lipids and contains coenzymes. The fibrils of the axial filament contain adenosine triphosphatase required for energy transfer and hence ensure spermatozoal motility.

I.7.2. Immunological functions of seminal plasma

The main functions of seminal plasma (reviewed by Alexander and Anderson, 1987 and Kelly, 1997[a]) include:

i) Facilitating spermatozoal transport.

ii) Prevention of a rapid loss of spermatozoa from vagina by providing a coagulating system.

iii) Provision of an energy source (fructose, glucose) and motility stimulant for spermatozoa.

iv) Serving as a highly buffered medium to overcome the acidic pH of the vagina.

v) Suppression of the host immune system by presence of immunosuppressive components such as prostaglandins, prostasome, polyamines, transforming growth factor (TGF)-β and complement inhibitors.

The immunological functions of the components of seminal plasma, in particular prostaglandins are addressed below.

i) <u>Prostaglandins</u>

It is known that human and primates have high levels of prostaglandins in semen at a concentration some 10^8 times more than that of peripheral blood (reviewed by Kelly[a], 1997). Prostaglandins in human seminal plasma include

four main species, 19-hydroxy prostaglandin E (19-OH PGE) 1, 19-OH PGE2, PGE1 and PGE2. Prostaglandins, which are released by macrophages together with stimulatory agents and other suppressive agents, have immunomodulatory effects. For instance, PGE2 has been shown to have a pro-inflammatory role and activates/matures cultured DCs (monocyte-derived DCs) and subsequently induces IL-12 production by DCs (Rieser *et al*, 1997; Rieser *et al*, 1998; Portanova *et al*, 1996). In contrast to this pro-inflammatory activity of PGE2, PGE2 is also known to inhibit the production of pro-inflammatory cytokines by lipopolysacharide (LPS)-activated macrophages (Strassmann *et al*, 1994). PGE2 has also been shown to inhibit IL-12 production (Kraan *et al*, 1995; Kelly *et al*, 1997[a]) and stimulate IL-10 production in whole blood culture (Strassmann *et al*, 1994; Kelly *et al*, 1997[a]). This cytokine switch to a Th2-type may induce anergy or peripheral tolerance (Groux *et al*, 1996). Spermatozoa are allogeneic but whether or not they exhibit MHC antigens on their surface is controversial (Vince *et al*, 1995; Schaller *et al*, 1993; Hutter and Dohr, 1998). The cytokine switch induced by prostaglandins in seminal plasma could result in tolerance to spermatozoal antigens in female reproductive tract and may also influence viral infection. Immunosurveillance and killing mechanisms might be compromised by repeated exposure to prostaglandins in semen by inducing T cell anergy (Groux *et al*, 1996) and inhibition of cytotoxic responses.

ii) Prostasomes

Prostasomes are submicron organelles, enclosed in a lipid membrane (Ronquist and Brody, 1985). Their known immunosuppressive effects are the inhibition of T cell proliferation (Kelly *et al*, 1991) and modulation of phagocytic activity (Skibinski *et al*, 1992).

iii) Polyamines

Polyamines have been found to inhibit nitric oxide production, which is required for killing and phagocytic action in monocytes (Szabo *et al*, 1994) and have also been shown to inhibit pro-inflammatory cytokine production (Zhang *et al*, 1997).

iv) Complement inhibitors

Complement inhibitors in semen include decay accelerating factor (DAF; CD55), which promotes C3 breakdown (Rooney *et al*, 1993[a]), membrane co-factor protein (CD46) which inactivates C3 (Simpson and Holmes, 1993) and protectin (CD59) which binds to spermatozoa to provide protection from complement attack (Rooney *et al*, 1993[b]). Complement inhibitors have also been shown to be incorporated into the HIV envelope and induce resistance to complement attack (Saifuddin *et al*, 1995).

I.7.3. HIV-1 in semen

Semen is considered to be an important vehicle for the sexual spread of HIV-1 and thus the level of virus in semen would be an important determinant in

the sexual transmission of HIV-1. Since the first quantitation of HIV-1 in semen by Borzy and co-workers in 1988 using reverse transcriptase activity assays in a 'virus pellet' obtained by differential sucrose density centrifugation of cell-free seminal plasma from symptomatic individuals (Brozy *et al*, 1988), many researchers have quantitated HIV-1 RNA levels in semen (Rasheed *et al*, 1995; Liuzzi *et al*, 1996; Liuzzi *et al*, 1995; Gupta *et al*, 1997; Coombs *et al*, 1998; Dyer *et al*, 1998). Some researchers have detected HIV-1-infected seminal non-sperm cells (Quyale *et al*, 1997; Vernazza *et al*, 1996; Baccetti *et al*, 1991; Bagasra *et al*, 1988; Bagasra *et al*, 1990). Therefore, HIV-1 is present in semen both as cell-free virus and cell (seminal non-spermatozoal cell)-associated virus. However, the possibility that HIV-1 is carried by the spermatozoa themselves has been suggested. Several studies have been carried out to investigate the presence of HIV-1 DNA in spermatozoa and whether spermatozoa express CD4. A number of groups have presented data suggesting that HIV attaches to and infects spermatozoa (Bagasra *et al*, 1988; Bagasra *et al*, 1994; Baccetti *et al*, 1994; Scofield, 1992; Scofield *et al*, 1994; Gobert *et al*, 1990), possibly via a CD4-like molecule (Ashida and Scofield, 1987; Bagasra *et al*, 1994; Scofield *et al*, 1992; Scofield *et al*, 1994; Gobert *et al*, 1990). Some groups have failed to detect CD4 expression on sperm (Wolff and Anderson, 1988[a]; Wolff and Anderson, 1988[b]; El-Demiry *et al*, 1986) and could not detect HIV-1 particles or proviral sequences in sperm cells (Mermin *et al*, 1991; Van Voorhis *et al*, 1991; Quayle *et al*, 1997). CD4 or CD4-like molecules on spermatozoa could play an important role in infection of spermatozoa with HIV-1 since CD4 is

the primary cell receptor for HIV-1. Also binding of sperm to somatic cells via HLA-DR (Ashida and Scofield, 1987; Scofield *et al*, 1992) carries potential significance for HIV transmission, because HLA-DR is present on mucosal cells within the primate female reproductive tract, many of which are susceptible to HIV infection *in vitro* (Langhoff *et al*, 1991; Scofield *et al*, 1992). It has also been shown that carbohydrates appear to be critical for sperm binding to HLA-DR and thus sulphated carbohydrates such as heparin, dextran sulphate, and chondriotin sulphate, could enhance adhesion of sperm to HLA-DR, possibly by stabilising the sperm-HLA-DR interaction (Scofield *et al*, 1992). Technical differences in HIV-1 detection and sperm preparation methods, or contamination of spermatozoal preparations with seminal non-sperm cells, may have resulted in discrepancies in results obtained by different groups. The data obtained in this study are presented in chapter III.

Molecules other than CD4 have been identified on spermatozoa, which may play a role in binding of HIV-1. Galactosylceramide or a related compound is present on the surface membrane of the mid-piece and equatorial segment of human spermatozoa (Baccetti *et al*, 1994) and has been shown to be an essential component of the neural receptor for HIV-1 gp120 (Bath *et al*, 1991; Harouse *et al*, 1991). Glycolipid molecules, most likely galactosyl-alkyle-glycerol which is structurally similar to galactosylceramides, has been detected on the surface of the mid-piece of spermatozoa and on the membrane of immature germ cells, preferentially in the spermatogonia. Furthermore, it has been demonstrated that these sperm glycolipids are capable of binding to

gp120 (Brogi *et al*, 1995). Since the mid-piece of sperm contains mitochondria that are responsible for generating energy for sperm motility, it may be possible that HIV-1 infection results in disrupted mitochondrial function leading to sperm immotility. Such data suggest that glycolipids may function as HIV receptors and this may explain the inhibition of spermatogenesis observed in AIDS patients (Martin *et al*, 1991). However, whether galactosylceramide functions as a receptor for HIV infection of spermatozoa, has not been confirmed.

Semen appears to be an isolated reservoir of HIV-1. There is evidence that seminal HIV may not arise from the same reservoir as peripheral blood:

i) HIV-1 viraemia in semen does not always correlate with that in blood plasma (Rasheed *et al*, 1995; Liuzzi *et al*, 1996; Gupta *et al*, 1997; Coombs *et al*, 1998) or with blood CD4 cell count (Gupta *et al*, 1997; Liuzzi *et al*, 1996).

ii) Genetic analysis of envelope (Zhu *et al*, 1996; Delwart *et al*, 1998) and protease (Kiessling *et al*, 1998) genes of HIV-1 isolates from paired specimens of blood and semen have revealed significant divergences of the two viral populations.

iii) Genetic characteristics of envelope gene sequences of sexually transmitted virus differ from those in the blood of the transmitter (Zhu *et al*, 1996).

iv) Detection of culturable HIV-1 in semen correlated strongly with HIV RNA levels in cell-free seminal plasma but only weakly with HIV

RNA levels in blood plasma or with CD4 cell count (Liuzzi *et al*, 1996; Coombs *et al*, 1998).

The means by which virus may gain entry to semen is not yet understood. Possibilities include that virus arises from the site from which the seminal leukocytes originated ('Hypothesis 1'), or alternatively that virus enters from another reservoir and infects seminal leukocytes within the seminal fluid ('Hypothesis 2'). The origin of leukocytes in semen is not yet well defined but these cells may arise from within the germ cell compartment. This hypothesis is supported by the following observations:

i) Vasectomy reduces seminal leukocyte numbers (Olsen and Shields, 1984)

ii) Leukocyte numbers have a direct correlation with sperm with ideal morphology (Kiessling *et al*, 1995; Tomlinson *et al*, 1993). One proposed role of leukocytes in the germ cell compartment is to eliminate defective germ cells (Yeung *et al*, 1994).

Evidence against hypothesis 1 comes from the fact that the testis and epididymis (fig.I.7) are isolated from routine immune surveillance mechanisms by a combination of anatomic barriers (Holash *et al*, 1993) and Fas-FasL interactions (Griffith *et al*, 1995) thereby protecting spermatozoa which are highly immunogenic to their host. Vasectomy, which eliminates proximal sources of HIV from germ cells and leukocytes and secretions from the testes, epididymis and vas deferens, has minimal impact on the infectivity of semen (Krieger *et al*, 1998), suggesting that germ cells are not a source of

HIV. This study also suggests that cell-free and cell-associated HIV arises distal to the vas deferens (e.g. in seminal vesicles, prostate, urethra or Cowper's gland). HIV has been detected in the pre-ejaculate which is produced by the urethral and Cowper's gland (Ilaria *et al*, 1992). Granulocytes, macrophages and T lymphocytes (both CD4 and CD8) have been found in pre-ejaculate (Pudney *et al*, 1992). The immunosuppressive properties of seminal vesicle and prostate fluids supports the hypothesis that these organs may be sites of immune-privileged HIV-infected cells. Furthermore, $CD4^+$ T cells are more abundant in the normal prostate compared with $CD8^+$ T cells (Ball *et al*, 1982). Taken together it seems that virus may gain entry to semen from the peripheral circulation via accessory organs (eg. prostate and seminal vesicles). Variations in seminal viral burden may be influenced by prostatitis or urethritis, which in turn could increase leukocyte numbers in the semen-producing organs and consequently the semen viral burden. Infection of the accessory organs may also result in compromised immunologic control mechanisms in germ cell compartments (which is protected from HIV infection early in the disease) and hence may lead to HIV infection of $CD4^+$ cells in germ cell compartments.

I.7.4. 'Sperm-washing'

'Sperm washing' is a technique used to improve the quality of sperm before intra-uterine insemination or *in vitro* fertilisation. It has been used for the management of infertility resulting from cervical or male infertility factors

and was first employed in 1973 to remove sperm antibodies (Halim *et al*, 1973).

HIV is present in seminal fluid as both free-virus and non-sperm cell-associated virus as described above (I.5.3). Thus sperm washing may be used as a means of minimising the exposure of an uninfected female partner of an HIV^+ male to potentially infected material, by reducing the free-virus and the HIV-infected cells. This application of 'sperm-washing' for HIV-discordant couples wishing to have children was first described in 1992 by Semprini *et al* (Semprini *et al*, 1992). To conceive, such HIV-discordant couples (the man is HIV positive and the woman is HIV negative) must abandon condom-protected intercourse and risk HIV transmission to the woman and to her child. 'Sperm-washing' as a risk reduction program has been a controversial area. It has been suggested that the risk of transmission is sufficiently low that such couples may attempt natural conception (Mandelbrot *et al*, 1997). 92 HIV negative women and their HIV positive partners received pre-conceptual counselling on the risk of transmission and were advised to pinpoint ovulation to reduce the risk. Two women seroconverted at seven months of pregnancy and another two post-partum, with seroconversions restricted to couples with inconsistent condom use. In contrast, Semprini's group have already carried out 1690 inseminations in 543 couples resulting in almost 240 pregnancies with no seroconversion in mothers or the resulting babies (Semprini *et al*, 1999, personal communication). Based on this clinical data, 'sperm-washing' appears to be a method of reducing the risk of

heterosexual transmission in HIV-discordant couples wishing to have children. However, the safety of sperm-washing has only been evaluated by the relatively insensitive method of antibody detection of viral antigen, p24, on the purified spermatozoa (Semprini *et al*, 1992). This thesis investigated, for the first time, the presence of both viral RNA and proviral DNA in the purified spermatozoa and the expression of receptors used for viral entry on these cells.

I.8. AIM OF THESIS

Understanding mechanisms of sexual transmission of HIV-1 is of importance in designing vaccine against HIV-1. Semen is considered to be an important vehicle in transmission of STDs including HIV-1 and immature DCs in the mucosa (LCs) are suggested to be the first cells to become infected. Therefore aim of this thesis is to investigate the role of semen in HIV transmission by answering following four questions:

i) Where does virus reside in seminal fluid?

ii) Does 'sperm-washing' really reduce risk of HIV transmission in HIV-discordant couples who wish to have children?

iii) What effect does seminal plasma have on DC phenotype and function?

iv) What effect does seminal plasma have on HIV infection of DCs?

II

MATERIALS AND METHODS

Suppliers for each reagent are listed in the section II.8 and suppliers for equipment are indicated where appropriate in the text.

II.1. SUBJECTS

Both semen and blood samples from HIV-1 positive patients were donated from HIV-1 positive men attending the St. Stephen's Clinic, Chelsea and Westminster Hospital, London. Control semen samples were donated from HIV negative donors attending the fertility clinic at the Chelsea and Westminster Hospital and from laboratory workers. Control blood samples were obtained from laboratory workers. For propagation of virus (II.6.1) buffy coat from HIV negative individuals was obtained from the North London Blood Transfusion Centre, Colindale, London, UK. The number of samples for each study is indicated individually in appropriate figures. Ethical approval for the studies was granted by the Riverside Research Ethics Committee. All the samples from HIV-1 positive patients were prepared in a containment level 3 laboratory.

II.2. SAMPLE PREPARATION AND CELL CULTURES

II.2.1. Peripheral Blood Mononuclear Cell separation

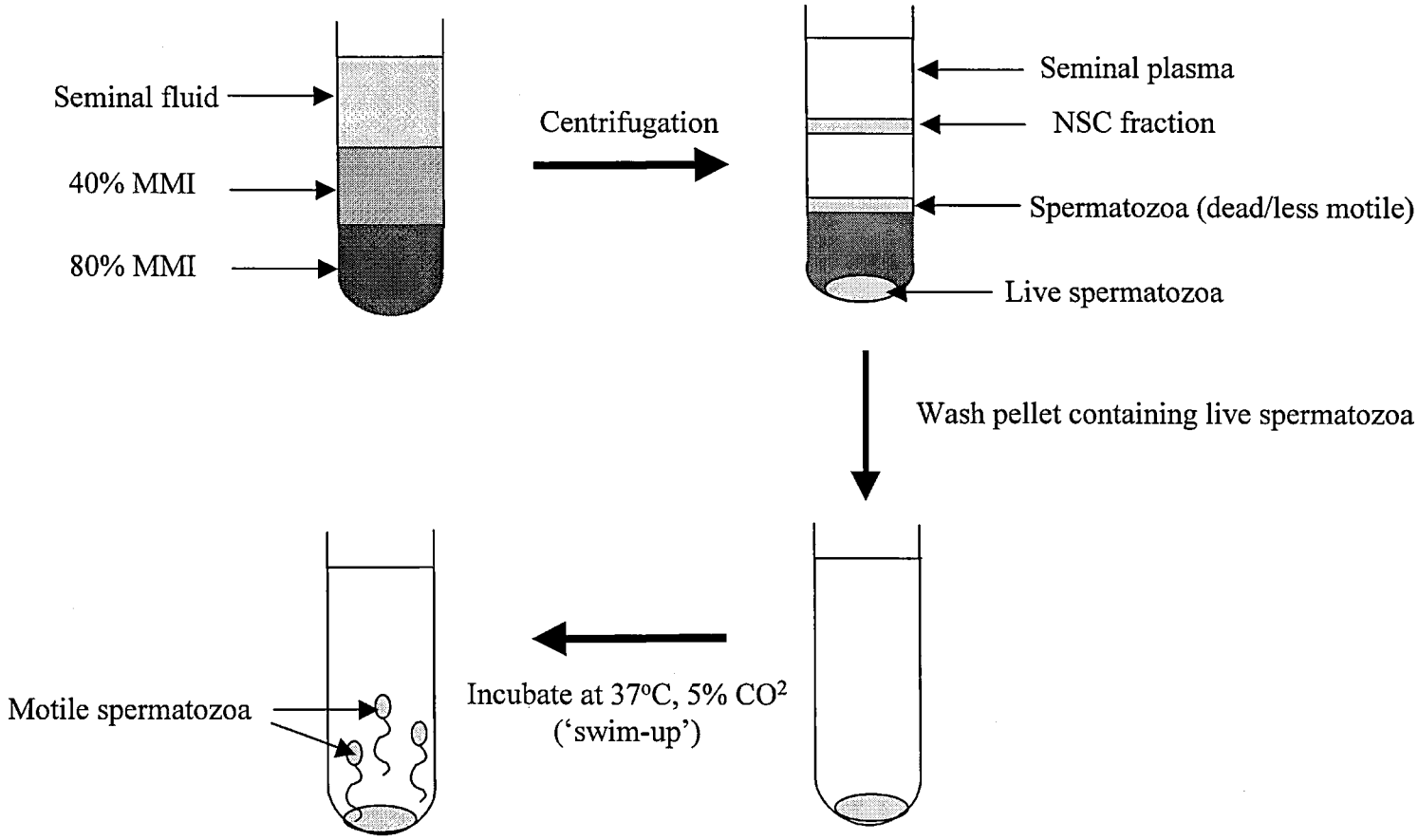
Blood samples were collected intravenously into vacutainers containing ethylenediamine tetraacetic acid (EDTA) or sodium heparin (Becton Dickinson, Oxford, UK). Plasma and peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation over Histopaque. The blood was overlaid on Histopaque and centrifuged at 600g for 30 minutes at room temperature. Plasma was stored at -70°C until use. The interface containing PBMCs was transferred into a fresh universal tube and washed twice in wash medium [serum-free RPMI1640 medium supplemented with 2mM L-glutamine, 50U/ml of penicillin, 50μg/ml of streptomycin and 1.5μg/ml of amphotericine B] by centrifugation at 400g for 10 minutes at room temperature. The viable cells were counted by 'trypan blue exclusion'.

II.2.2. Semen Separation

Semen samples were donated by masturbation into a sterile plastic container. The samples were kept at 37°C until processing and all specimens were processed within 2 hours of donation. Unfractionated semen samples were diluted 1:4 in pre-warmed (37°C) complete Earl's Balanced Salt (EBS) medium [EBS solution supplemented with 1mM pyruvic acid and 1mM lactic

acid and pH adjusted to 7.2 using concentrated HCl]. A fraction of the diluted whole semen was stored at -70°C. Seminal plasma, spermatozoa and seminal non-sperm cells (NSCs) were prepared by differential gradient centrifugation over 80% and 40% Medicol Medium Isotonic (MMI) at 400g for 30 minutes at room temperature. The semen sample was separated into 4 fractions: seminal plasma, upper interface containing NSCs, lower interface containing dead or less motile spermatozoa and the pellet containing mainly live and motile spermatozoa (fig.II.1). The cells from both interfaces and the pellet were washed twice in complete EBS medium by centrifugation at 400g for 10 minutes at room temperature. Following the final wash the spermatozoal pellet was overlaid with 3ml of medium and incubated at 37°C, 5% CO_2 for 20 minutes to allow motile spermatozoa cells to swim up. The supernatant containing motile spermatozoa was collected. In some experiments, the spermatozoa fraction at the 40% / 80% interface was also collected. This fraction should contain mostly dead spermatozoa, however when treated in the same way as the pellet from the gradient a number of viable 'swim-up' spermatozoa were recovered. The seminal plasma fraction was filtered through a 0.2μm filter (Sartorius, Surrey, UK) and stored at -70°C. Seminal plasma samples from six HIV-negative individuals were mixed together to make a pool of seminal plasma and stored at –70°C. This 'seminal plasma pool' was used to pulse monocyte-derived dendritic cells (MDDCs) as described in II.2.6 for studies presented in chapter 4 and chapter 5. For some experiments, lipids from seminal plasma (HIV-negative) were extracted at low pH using reverse

Fig. II.1. Semen separation by differential gradient centrifugation over 80% and 40% Medicol Medium Isotonic (MMI)



phase columns as previously described (Kelly *et al*, 1997[a]). Seminal plasma was adjusted to pH4 using concentrated HCl prior to passing through C-18 reverse phase columns (SepPak, Waters, UK) which were pre-treated with methanol and washed with distilled water. The lipid-extracted seminal plasma was filtered through a 0.2μm filter.

II.2.3. Freezing and Thawing Cells

Cells for DNA and RNA extraction were snap frozen at -70°C. Cells for culture were re-suspended in 0.5ml of freezing mix [90% FCS and 10% Diemethyl sulphoxide (DMSO)] at a concentration of $5x10^6$/ml and were frozen in an isopropanol-insulated chamber ('Mr. Frosty') placed at -70°C, over night. The next day, the cells were transferred to and stored in liquid nitrogen. Frozen vials were thawed quickly in a 37°C water-bath and immediately diluted with cold R10 medium [serum free RPMI1640 supplemented with 2mM L-glutamine, 50U/ml of penicillin, 50μg/ml of streptomycin, 1.5μg/ml of amphotericine B and 10% FCS]. The cells were washed twice at 400g for 7 minutes at room temperature, resuspended in R10 medium and incubated at 37°C, 5% CO_2.

II.2.4. Separation of $CD14^+$ Cells Using MicroBeads

$CD14^+$ cells were positively selected from PBMCs using CD14 MicroBeads according to the manufacturer's instructions. Briefly, PBMCs were washed in cold MiniMACS buffer [0.2mM EDTA, 0.5% FCS in PBS]. 10^7 cells in 80µl of MiniMACS buffer were incubated with 20µl of CD14 MicroBeads at 6-12°C for 15 minutes with occasional mixing. The cells were washed once by centrifugation at 400g for 10 minutes at 6°C and then resuspended in 1ml of cold MiniMACS buffer. The cell suspension was applied to a pre-wet MS+ column (Miltenyi Biotec) which was placed in the magnetic field of a MACS separator (Miltenyi Biotec). The magnetically labelled $CD14^+$ cells were retained in the column while the unlabelled $CD14^-$ cells ran through. The $CD14^-$ cells were washed off from the column twice with 500µl of MiniMACS buffer. After removal of the column from the magnetic field, the magnetically retained $CD14^+$ cells were eluted as for the positively selected cell fraction.

II.2.5. Generation of Dendritic Cells from $CD14^+$ Monocytes

3ml of $CD14^+$ cells at a concentration of 10^6/ml in dendritic cell (DC) culture medium [R10 medium supplemented with 50ng/ml of GM-CSF and 20ng/ml of rIL-4] were placed in a 6-well tissue culture plate (Greiner, Gloucestershire, UK) and cultured at 37°C, 5% CO_2 for 5 days. 50ng/ml GM-CSF and 20ng/ml rIL-4 were added every 3 days and cultured for 5 days to obtain $CD14^+$

monocyte-derived DCs (MDDCs). Phenotype of MDDCs after 5 days of culture is shown in fig.II.2.

II.2.6. Pulsing of dendritic cells with Seminal Plasma, PGE2 or 19-OH PGE2 and/or TNF-α

Seminal plasma (at a final concentration of 0.1% and 1%; a pool of seminal plasma as described in II.2.2 was used), prostaglandin E2 [PGE2 (at a final concentration of 0.0071μM, 0.071μM and 0.71μM)] or 19-hydroxy PGE2 [19-OH PGE2 (at a final concentration of 0.0353μM, 0.353μM and 3.53μM)] was added to MDDC cultures at day 5 and incubated overnight at 37°C, 5% CO_2. In some experiments, cells were pulsed overnight with 200U/ml of TNF-α alone or together with seminal plasma, PGE2, or 19-OH PGE2. The next day, the cells were washed twice in R10 medium by centrifugation at 400g for 10 minutes at room temperature before setting up further experiments. The optimum concentration of TNF-α (200U/ml) was determined experimentally and the data is shown in chapter 4. The amounts of PGE2, 19-OH PGE2 used were calculated as follows.

a) <u>PGE2 (Calbiochem)</u>

Molecular weight of PGE2 = 352.5

PGE2 in dry pellet (1mg) was dissolved in 1ml of ethanol to give concentration of 2.84mM (concentration of PGE2 stock).

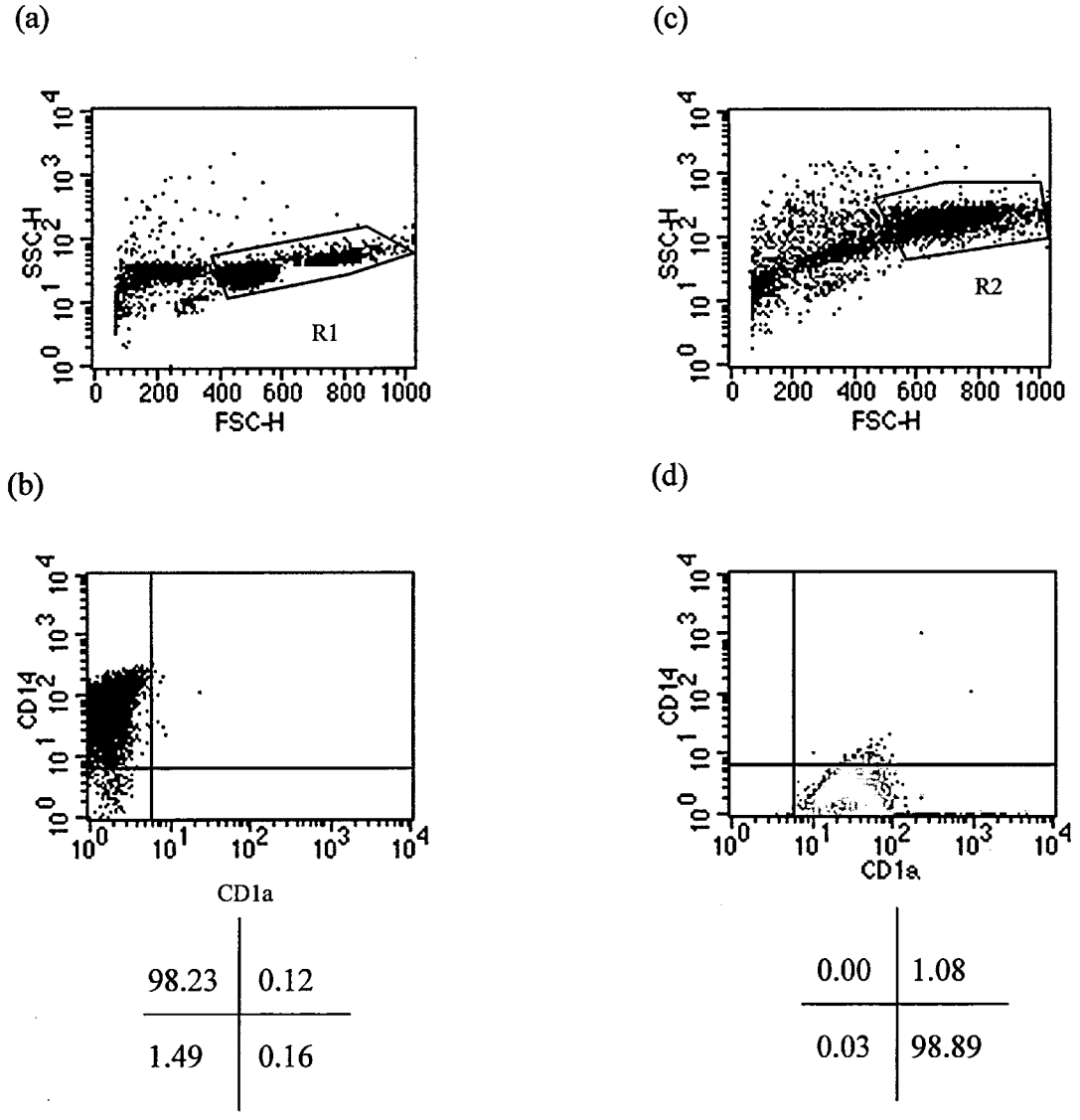


Fig.II.2. Flow cytometric analysis showing the CD14$^+$ monocyte population after CD14$^+$ cell separation using MicroBeads and the CD1a$^+$ immature DC population after 5 days of culture in the presence of IL-4 and GM-CSF.

CD14$^+$ monocytes were separated from PBMCs using MicroBeads as described in II.2.4 (a). The purified cells were stained with monoclonal anti-CD14 antibody (c) and were cultured for 5 days in the presence of IL-4 and GM-CSF (b). At day 5 the cells were stained with monoclonal anti-CD1a antibody (d).

Average concentration of PGE2 in seminal plasma is 25μg/ml (Kelly *et al*, 1997[a]) which is equivalent to 71μM (i.e. $\frac{25}{352500} = \sim 7.1 \times 10^{-5}$M or 71μM).

Therefore, 0.1% seminal plasma contains 0.071μM and 1% seminal plasma 0.71μM of PGE2. The volume of PGE2 stock to be added to MDDC cultures to give a final concentration equivalent to 0.1% or 1% seminal plasma was calculated using following equation:

Volume of PGE2 stock to add $= \frac{A}{2840}$ x 0.071 [(for 0.1% seminal plasma) or

0.71 (for 1% seminal plasma)]

(where A is final volume of MDDC culture)

(b) 19-OH PGE2 (Cayman)

Molecular weight of 19-OH PGE2 = 368.5

Concentration of 19-OH PGE2 solution was 500μg/ml which is equivalent to 1.36mM.

Average concentration of 19-OH PGE2 in seminal plasma is 130μg/ml (Kelly *et al*, 1997[a]) which is equivalent to 353μM.

[i.e. $\frac{130}{368500} = \sim 3.53 \times 10^{-4}$M or 353μM]

Therefore, 0.1% seminal plasma contains 0.353μM of 19-OH PGE2 and 1% seminal plasma contains 3.53μM of 19-OH PGE2. The volume of 19-OH PGE2 stock to be added to MDDC cultures to give a final concentration which is equivalent to 0.1% or 1% seminal plasma was calculated using following equation:

Volume of PGE2 stock to add $= \frac{A}{1360}$ x 0.353 [(for 0.1% seminal plasma) or

3.53 (for 1% seminal plasma)]

(where A is final volume of MDDC culture)

II.2.7. Allogeneic Mixed Lymphocyte Reaction

MDDCs treated with seminal plasma as described in II.2.6 were used as stimulators in an allogeneic mixed lymphocyte reaction (MLR). Fresh PBMCs were used as responder cells. An increasing number of irradiated (3,000 rads for 9 minutes) MDDCs (0 to 8,000 cells/well) and 10^5 responder cells were added to a 96-well U-bottomed tissue culture plate (Greiner) in R10 medium. After 4 days, the cells were pulsed with [^{3}H]-thymidine and harvested on a filtermat (Wallac) the next day (16-hour pulse with 0.5μCi/well). Proliferation was measured as [^{3}H]-thymidine incorporation. Results are shown as mean stimulation indices (SI) with standard error (SE) throughout this thesis in order to overcome difficulties in summarising results from different samples due to a large variance in mean counts per minute (CPM) readings between samples. Representative graphs of each experiment are also presented as a mean CPM with SE. The number of experiments performed was stated individually in appropriate figures presented throughout this thesis and each experiment was set up in triplicate.

II.2.8. Recovery of allogeneic T cell proliferation

The MDDCs were pulsed with seminal plasma overnight as described in II.2.6. The cells were washed twice, irradiated and used to set up allogeneic MLRs ($4x10^4$ MDDCs in 0.5ml + 10^6 responder cells in 0.5ml of R10 medium) in a 24-well tissue culture plate (Greiner). At day 3, the cells were harvested, washed twice, counted ('trypan blue exclusion') and then employed as responders in a second MLR. Untreated fresh MDDCs from the same donor as those in the first MLR were used as stimulators. The second MLR was set up in a 96-well tissue culture plate as follow: i) untreated MDDCs only, ii) 20 units of rIL-2 alone or iii) both MDDCs and rIL-2. In all conditions described 10^5 cells from the primary MLR were added. At day 6, the cells were pulsed with 0.5μCi/well of [^{3}H]-thymidine and harvested next day. The results were shown as mean SI with SE. This experiment was performed three times and each experiment was set up in triplicate.

II.3. FLOW CYTOMETRY

II.3.1. Surface Marker Staining

Cells were washed once with MiniMACS buffer by centrifugation at 400g for 10 minutes at 6°C. 1-$10x10^5$ cells in 100μl of MiniMACS buffer were

incubated with appropriate antibodies [1μg/10^6 cells (table II.1)] on ice for 30 minutes in dark. Unbound antibodies were washed off by centrifugation at 400g for 10 minutes at 6°C. Stained cells were re-suspended and fixed in 500μl of FACS Fix [1% (or 2% for samples from HIV positive patients) paraformaldehyde in MiniMACS buffer]. When indirect immunofluorescence or multiple colour staining was required the subsequent antibody was added to the cells after washing off unbound first or second layer of antibodies. The cells were then analysed within 24 hours on a Becton Dickinson FACScalibur using Cellquest software.

II.3.2. Intracellular Cytokine Staining

Cells to be stained for intracellular cytokines were incubated with 10μg/ml of Brefeldin A (BFA) overnight. Cells without BFA as control were also set up. When required, 1μg/ml of Ionomycin and 25ng/ml of Phorbol 12-Myristate 13-Acetate (PMA) were added to stimulate cytokine production to the culture in conjunction with BFA. Following all surface staining as described in II.3.1, the cells were permeabilised using FACS Permeabilising Solution. The FACS Permeabilising Solution (10x) was diluted in distilled water to make a working concentration (1x). 500μl of the diluted FACS Permeabilising Solution was added to the cell pellet, vortexed and incubated for 10 minutes at room temperature in the dark. The permeabilised cells were washed in MiniMax buffer containing 0.1% Sodium Azide (NaN_3) prior to staining with specific antibodies for cytokines. Intracellular cytokine staining procedures were

Table II.1. Antibodies used for flow cytometric analysis

Marker	Clone	Isotype	Fluorescence
CD1a (Harlan Sera-Lab) (Serotec)	NA1/34HLK NA1/34	Mouse IgG2a Mouse IgG2a	FITC Pure
CD3 (Sigma)	UCHT-1	Mouse IgG1	FITC/ Q-red
CD4 (Sigma) (Dako)	Q4120 MT310	Mouse IgG1 Mouse IgG1	PE/Q-red PE
CD8 (Sigma)	UCHT-4	Mouse IgG2a	PE
CD14 (Sigma)	UCHM-1	Mouse IgG2a	PE
CD80 (Pharmingen)	BB1	Mouse IgM	FITC
CD86 (Pharmingen)	2331 (FUN-1)	Mouse IgG1	PE
HLA-DR (Serotec) (Serotec) (Harlan Sera-Lab)	TAL.IB5 TU36 B-F1	Mouse IgG1 Mouse IgG2b Mouse IgG1	RPE-Cy5 RPE-Cy5 FITC
HLA-DQ (Sigma)	HK19	Mouse IgG2b	FITC
CCR5 (R&D)	45502.111	Mouse IgG2b	FITC
CXCR4 (NIBSC)	44708.111	Mouse IgG2a	Pure
Streptavidin (DAKO)	-	-	PE
Sperm (Chemicon)	Polyclonal	-	Pure
Sheep-IgG (Sigma)	-	-	FITC
IFN-γ (Pharmingen)	4S.B3	Mouse IgG1	PE
IL-4 (Pharmingen)	MP4-25D2	Rat IgG1	FITC
IL-10 (Pharmingen)	JES3-9D7	Rat IgG1	PE
IL-12 (Pharmingen)	C11.5	Mouse IgG1	PE

FITC=fluorescein-isothiocynate, PE=R-Phycoerythrin, Q-red=Quantum red, RPE-Cy5= R-Phycoerythrin cychrome 5

carried out at room temperature. Stained cells were analysed within 24 hours of staining on a flow cytometer (Becton Dickinson FACScalibur using Cellquest software).

II.4. NUCLEIC ACID EXTRACTION

II.4.1.Tri Reagent™

Following the manufacturer's instructions, 10^6 cells were homogenised in 1ml of Tri Reagent™ (a mixture of guanidine thiocyanate and phenol in a mono-phase solution) and the mix was frozen at -70°C immediately, and thawed slowly on ice. 0.2ml of chloroform was added to the sample. The resulting sample was shaken vigorously and was allowed to stand for 5 minutes on ice followed by centrifugation at 12,000g for 10 minutes at 4°C. This resulted in separation of the mixture into 3 phases: a red organic phase containing protein, an interface containing DNA, and a colourless upper aqueous phase containing RNA.

II.4.1.1. RNA Isolation

The aqueous phase containing RNA was transferred to a 1.5ml-eppendorf tube (Elkay, Hampshire, UK). 0.5ml of isopropanol was added to the sample and

mixed. The resulting mixture was left to stand for 5 minutes at 4°C and was centrifuged at 12,000g for 10 minutes at 4°C. The RNA pellet was washed with 1ml of 75% ethanol: the sample was mixed well and centrifuged at 2,000g for 5 minutes at 4°C. The washed RNA pellet was air-dried and re-suspended in 30µl of nuclease-free water and stored at -70°C. The concentration of RNA was measured using a spectrophotometer (II.4.4). RNA was prepared in a cold room.

II.4.1.2. DNA Isolation

The interface containing DNA was transferred to a 1.5ml-eppendorf tube. 0.3ml of absolute ethanol was added to the sample and mixed. The resulting mixture was left to stand for 3 minutes at 4°C and then centrifuged at 2,000g for 5 minutes at 4°C. The DNA pellet was washed twice in Wash Solution [0.1M sodium citrate, 10% ethanol]. During each wash, the DNA pellet was allowed to stand for 30 minutes at 4°C with occasional mixing. After the final wash, the DNA pellet was re-suspended in 1.5ml of 75% ethanol and was left to stand for 15 minutes at 4°C followed by centrifugation at 2,000g for 5 minutes at 4°C. The washed DNA pellet was air-dried and resuspended in 100µl of nuclease-free water. If the DNA pellet was difficult to resuspend a larger volume of water was added or the DNA suspension was heated to 80°C until complete resuspension was achieved. The concentration of DNA was

measured using a spectrophotometer (II.4.4) and 1μg/μl DNA stock was prepared and stored at -20°C until use.

II.4.2. RNAzol™ B

Following manufacturer's instructions, 5-10x10^5 cells were homogenised in 0.2ml of RNAzol™ B. Chloroform (one tenth volume of the homogenate) was added to the lysed cell suspension. The mixture was shaken vigorously for 15 seconds and allowed to stand for 5 minutes at 4°C followed by centrifugation at 12,000g for 15 minutes at 4°C. This resulted in the separation of the mixture into 2 phases: a lower blue phenol:chloroform phase and a colourless aqueous phase containing RNA in 50% of the initial volume of RNAzol™ B. This aqueous phase was transferred to a 1.5ml-eppendorf tube. An equal volume of isopropanol was added to the tube and mixed. This mixture was incubated at 4°C for 15 minutes followed by centrifugation of the mixture at 12,000g for 15 minutes at 4°C. The RNA pellet was washed once with 1ml of 75% ethanol. The washed RNA pellet was air-dried, re-suspended in 30μl of nuclease-free water and stored at -70°C. The concentration of RNA was measured using a spectrophotometer (II.4.4).

II.4.3. NucliSens™ Lysis Buffer

All the reagents used in this part of the experiment were supplied by Organon Teknika and were used as per the manufacturer's instructions. 100µl of blood plasma; 100µl of seminal plasma (1:4 diluted); 100µl of unfractionated semen (1:4 diluted); 10^5-10^6 spermatozoa, or 10^5-10^6 NSCs were used to extract nucleic acid. The samples were added to 0.9ml of the lysis buffer provided (Tris/HCl, Triton X-100, 5M guanidine thiocyanate: GuSCN) and centrifuged for 30 seconds at 10,000g. 20µl of calibrator provided was added to the mixture which was then vortexed and centrifuged at 10,000g for 30 seconds. After mixing the silica suspension well, 50µl of the silica suspension was added to the sample mix. The resulting mixture was left to stand for 10 minutes at room temperature (with vortexing every 2 minutes) to allow the nucleic acids to be captured on the silica, and was then centrifuged at 10,000g for 30 seconds to pellet the nucleic acid bound silica. The silica-nucleic acid pellet was washed twice with 1ml of the wash buffer (Tris/HCl, 5M GuSCN) to remove DNase and RNase. The silica-nucleic acid pellet was then washed twice with 1ml of 70% ethanol to remove GuSCN and to precipitate nucleic acids. Finally 1ml of acetone was added. Following the acetone wash, the silica pellet was dried at 56°C for 10 minutes using a dry heating block (since acetone interferes with PCR reactions it is necessary to dry the pellet well). 50µl of the elution buffer (Tris/HCl) was added to the dried silica pellet, the resulting suspension was mixed well by vortexing and was left at 56°C for 10

minutes with occasional mixing to allow the silica bound nucleic acids to be released. Finally the nucleic acid was pelleted by centrifugation at 10,000g for 2 minutes at room temperature.

II.4.4. Spectrophotometer

The quantity of nucleic acid extracted was measured using a spectrophotometer. The RNA or DNA was diluted 1 in 100 using nuclease-free water and transferred to a cuvette which was placed in a spectrophotometer The spectrophotometer was calibrated based on a 'blank' (i.e. 1ml of nuclease-free water only). The optical density of the DNA and RNA was read at 260nm and 280nm. Calculation of nucleic acid purity was based on the ratio of OD_{260} to OD_{280}: if the ratio of OD_{260} to OD_{280} is above 1.5 for DNA and above 1.8 for RNA the nucleic acid prepared is regarded as pure.

Calculation of quantity of nucleic acid (μg/μl):

$$\frac{OD_{260} \times \text{Constant} \times \text{Dilution factor}}{1000}$$

(Constant =50 for DNA and 44 for RNA)

II.5. MOLECULAR ANALYSIS OF THE CELLS

II.5.1. Reverse Transcription-Polymerase Chain Reaction for CD4

The reverse transcription (RT) reaction mix [PCR buffer II, 4mM dNTP, 5mM $MgCl_2$, 2.5mM Oligo $d(T)_{16}$] was prepared on ice. 0.3μg of RNA template was added per 20μl RT reaction mix. This was heated to 80°C in a thermocycler (Perkin-Elmer; Techne) for 5 minutes to denature the dsRNA, cooled to 4°C and then placed on ice before adding MuLV reverse transcriptase (50 units/20μl RT reaction mix) and 20 units of RNase inhibitor. The complete RT reaction mix was returned to the thermocycler immediately and reverse transcription was performed as follows:

42°C 60 minutes (reverse transcription)
99°C 5 minutes (inactivation of the reverse transcriptase and denaturation of RNA-cDNA duplex)
Cool down to 4°C

The cDNA synthesised was kept at -20°C until use. PCR was performed with 2μl of the cDNA template per reaction as described in II.5.2.

II.5.2. Polymerase Chain Reaction for viral (HIV-1) DNA detection

The PCR mix [PCR buffer II for Ampli-*Taq* Gold DNA polymerase, or Stoffel buffer for Ampli-*Taq* DNA polymerase, Stoffel fragment, 1mM dNTP,

3.75mM $MgCl_2$, 1μM primer, and 2 units of Ampli-*Taq* Gold DNA polymerase or Ampli-*Taq* DNA polymerase, Stoffel fragment] was prepared on ice in a DNA-free clean room. 0.2μg of the DNA template for DNA PCR or 2μl of cDNA from reverse transcription (II.5.1) was added per 20μl reaction mix. Nested PCR was performed for HIV proviral DNA detection and β-globin primers were used as positive controls. For nested PCR 0.5μl of the first round PCR product was used as template. β-actin primers were used as a positive control primer for RT-PCR for CD4. The PCR reaction mix was heated to 95°C for 5 minutes to denature dsDNA (pre-PCR), cooled to 4°C and then transferred onto ice to add the enzyme, Taq DNA polymerase. The complete reaction mix was returned to the PCR thermocycler immediately and PCR was performed as follows:

95°C 12 minutes for Ampli-*Taq* Gold DNA polymerase

5 minutes for Ampli-*Taq* DNA polymerase

(pre-PCR denaturation/activation of Ampli-*Taq* Gold DNA polymerase)

95°C 1 minute (denaturation) ┐

Annealing for 1 minute* │ 40 cycles

72°C 1 minute (extension) ┘

72°C 10 minutes (extension)

Cool down to 4°C

*Annealing temperature for 1st round proviral DNA detection was 50°C and for nested reaction was 45°C. The annealing temperature for CD4 cDNA amplification was 56°C.

Primers for β-globin and HIV-1 *env* were quoted from Simmonds *et al* (1990) and synthesized by Perkin-Elmer. Primers for β-actin and CD4 were designed by Dr. M. Hope and synthesized by Gibco-BRL.

Table II.2. Primers

Gene	Primer sequence (5'-----3')	Product size (bp)
β-globin (1st round)	5' GGTTGGCCAATCTACTCCCAGG 3'	535
	5' GCTCACTCAGTGTGGCAAAG 3'	
β-globin (Nested)	5' ACACAACTGTGTTCACTAGC 3'	109
	5' CAACTTCATCCACGTTCACC 3'	
HIV-1 *env* (1st round)	5' TCAGGAAGGGGACCCAGAAATT 3'	501
	5' GATCCCATAGTGCTTCCTGCTGCT 3'	
HIV-1 *env* (Nested)	5' GGGGAATTTTTCTACTGTAAT 3'	304
	5' CTTCTCCAATTGTCCCTCATA 3'	
β-actin	5' TGGGTCAGAAGGATTCCTATGTGG 3'	465
	5' GTCGGCAATGCCAGGGTACATGG 3'	
CD4	5' ATAAAGATTCTGGGAAATCAGGGCTCC 3'	751
	5' TGCAACTTTCCTGTTTTCGCTTCAAGG 3'	

II.5.3. Quantitation of HIV-1 RNA Load Using NucliSens™

Nucleic acid was extracted as described in II.4.3 and used for quantitation of HIV-1 RNA load using the following procedure.

II.5.3.1. Amplification

All the reagents used in this part of the experiment were supplied by Organon Teknika and were used as per the manufacturer's instructions. 5µl of nucleic acid suspension was mixed with 10µl of primer mix provided and was left to stand at 65°C for 5 minutes to denature the nucleic acids and then placed at 41°C for 5 minutes to anneal the primers. 5µl of the enzyme mix (Avian myeloblastosis virus reverse transcriptase, Phage T7 RNA polymerase, and *E. coli* RNase H) was added to the nucleic acids/primers mix. The resulting mixture was gently mixed by rotating the tube and incubated at 41°C for 5 minutes for dsDNA template synthesis. The amplification mixture was centrifuged at 10,000g for 30 seconds and was incubated at 42°C for further 90 minutes to generate ssRNA product.

II.5.3.2. Detection

All the reagents used in this part of the experiment were supplied by Organon Teknika and were used as per the manufacturer's instructions. 5µl of the

amplificate and an appropriate amount of the detection buffer to give a dilution of 1 in 21 were mixed well. 5µl of the diluted amplificate was added to each tube containing 20µl of four oligo-beads/detection probe mix. The resulting amplificate/probe-bead mix was incubated at 41°C for 30 minutes to allow hybridisation of probe-bead with diluted amplificate. The hybridised RNA with probe-bead was detected by an electrochemical luminescence technique using an automated detection machine and HIV-1 RNA QT software.

II.5.4. Gel Electrophoresis

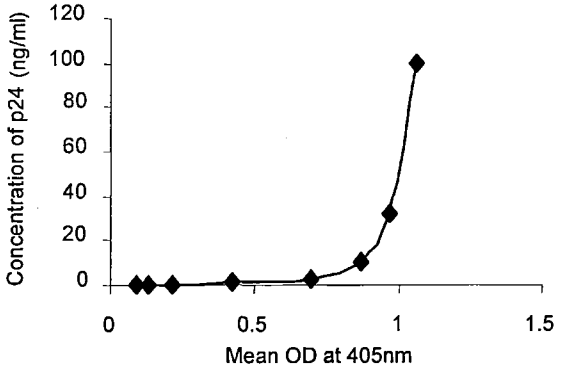
5µl of loading buffer [20% glycerol, 0.25% bromophenol blue, 0.1M EDTA in distilled water] was added to the 20µl of the PCR product and 10µl of the mixture was loaded to each well of a gel. The agarose gel which was prepared in Tris-Borate EDTA (TBE) buffer was placed in TBE buffer and run at 70V, 100mA for 30-40 minutes. Total RNA, genomic DNA, RT-PCR products, and PCR products were visualised on a 1% or 1.5% agarose gel in the presence of ethidium bromide under a transilluminator.

II.6. *IN VITRO* INFECTION OF DENDRITIC CELLS WITH HIV-1

II.6.1. Propagation of Virus

PBMCs from the buffy coat (North London Blood Transfusion Centre) were separated as described in II.2.1. The cells were stimulated with 10U/ml of rIL-2 and 10μg/ml of phytohaemaglutinin (PHA) in R15 medium [serum free RPMI1640, supplemented with 2mM L-glutamine, 50U/ml of penicillin, 50μg/ml of streptomycin, 1.5μg/ml of amphotericine B and 10%, 15% FCS] for 3 days. The PHA blasts from the two different donors were mixed and resuspended in R15 media at a concentration of $2x10^6$/ml. Viral stock (table II.2; kind gift from Prof. J. Weber and Dr S. Beddows, St. Mary's Hospital, London, UK) were added (>125 ID_{50}) to the PHA blasts and incubated at 37°C, 5% CO_2 for 2 hours. 3ml of R15 medium with 10U/ml of rIL-2 was then added to the culture and incubated overnight at 37°C, 5% CO_2. 10^7 of 3 day-old PHA blasts in 5 ml of R15 media containing rIL-2 were added to the culture. To maintain the viral cultures, half the volume of medium was replaced twice per week and fresh medium containing $3x10^6$ PHA blasts were added once a week. The virus in the supernatant was harvested by centrifugation at 400g and 0.2μm filtered. Aliquots of the viral stock were kept at -70°C for viral quantification (II.6.2) and for *in vitro* infection study. All the procedures were performed in class II hoods in containment level III facility. Concentrations of propagated virus were measured by p24 protein ELISA (II.6.2) and results are shown in fig.II.3. The p24 protein concentration was evaluated using 'Growth

(a)



Amount of p24 of standard control (ng)/ml	Mean OD at 405nm for standard control
0	0.093
0.0316	0.0136
0.1	0.0139
0.316	0.217
1	0.425
3.16	0.699
10	0.872
31.6	0.969
100	1.06

(b)

Viral isolate		Amount of p24 (ng)/ml
JRCSF	Day 3	0.6
	Day 7	0.8
	Day 13	1.5
PE106	Day 3	0.7
	Day 7	0.4
	Day 13	1.3
JW5	Day 3	0.5
	Day 7	0.6
	Day 13	0.5

Fig.II.3. Virus propgation: measurement of p24 protein production by ELISA

Virus was grown in PHA/IL-2-stimulated HIV-negative PBMCs (II.6.1). Virus in supernatants were harvested at different time points indicated and optical density (OD) at 405nm was read. Concentrations of p24 protein in each virus stock were evaluated from the standard curve (fig.II.3a) and are shown in fig.II.3b.

Function' (Excel 97) which allows predicted exponential growth by using existing data (i.e. standard curve that were plotted from the ELISA readings of known p24 protein concentration). The detection limit was 0.0316ng/ml. The highest concentration of p24 protein was obtained between 7 and 14 days of culture. For *in vitro*-infection studies the viral stocks were used at a final p24 protein concentration of 0.1ng/ml.

Table II.3. Viral isolates used to infect DCs.

Viral isolates	Properties
JRCSF	M-tropic or R5 strain, laboratory adapted
PE106	Dual-tropic or R5/X4 strain, primary isolate
JW5	T-tropic or X4 strain, primary isolate

II.6.2. p24 Enzyme Linked Immunosorbent Assay (ELISA)

The protocol was developed by Dr S. Beddows, St. Mary's Hospital, London, UK. The anti-p24 antibody (D7320) was diluted 1:100 in Coating Buffer (100mM $NaHCO_3$, pH adjusted to 8.5 using NaOH). 100µl of the diluted antibody was added to each well of a 96 well microtitre ELISA plate, covered and incubated overnight at room temperature. The plate was washed three times with 1x TBS [0.144M NaCl, 0.05% Tween 20, 25mM Tris, pH adjusted to 7.5 using concentrated HCl]. 200µl of Blocking Buffer [3% milk powder in 1x TBS] was added to each well and incubated at room temperature for 30

minutes and washed three times with 1x TBS. The samples to be assessed for p24 production were pre-treated with 0.1-0.2% Empigen (detergent) for 30 minutes at 56°C in a water-bath to inactivate HIV without destroying the antigenic epitopes. 100µl of the empigen-inactivated samples were added to each well, covered and incubated overnight at room temperature. The wells were washed six times with 1x TBS next day. The biotinylated anti-p24 antibody was diluted 1:100 in TMT/SS [2% milk powder, 20% lamb serum, 0.5% Tween 20 in 1x TBS]. 100µl of the diluted biotin-conjugated anti-p24 antibody was added to each well and incubated at room temperature for 2 hours. The plate was washed six times with 1x TBS. The streptavidin-AP was diluted to give 2 units/ml. 100µl of the diluted streptavidin-AP was added to each well, incubated at room temperature for 1 hour and washed again six times with 1x TBS. 0.1mg of the substrate (pNPP phosphatase substrate) in 100µl of Reaction Buffer [10mM Ethanolamine, 0.5mM $MgCl_2$, pH adjusted to 9.8 using concentrated HCl] was added to each well and incubated at room temperature in the dark until the optical density (OD) of the 100ng/ml control was more than 1.0. A p24 antigen control stock [(kind gift from Prof. J. Weber and Dr. S. Beddows, St. Mary's Hospital, London, UK) (1mg/ml)] was titrated in PBS/E/S (0.1% Empigen, 10% lamb serum in PBS) to give a standard curve (ie. 100ng/ml, 31.6ng/ml, 10ng/ml, 3.16ng/ml, 1ng/ml, 0.316ng/ml, 0.1ng/ml and 0.0316ng/ml) and PBS/E/S was used as a negative control. The plate was read at an OD of 405nm. Concentrations of p24 protein were evaluated from the standard curves which are obtained from OD readings of the p24 antigen

controls ('standard control'). The p24 antigen control ELISA was set up in duplicate and the standard curves were obtained from the mean OD readings of duplicate.

II.6.3. *In Vitro* Infection of Dendritic Cells with HIV-1

Virus was treated with DNase (ie. 50 units of RQ1 RNase-free DNase). The DNase was added to 1ml of virus suspension, mixed well and incubated at 37°C for 15 minutes on a dry-hot block (Techne). 100µl of the DNase treated viral supernatant was added to 1ml of the 5 day-old MDDC culture (10^6/ml; II.2.5) to give a final p24 protein concentration of 0.1ng/ml and incubated overnight at 37°C, 5% CO_2. 100µl of the DNase treated viral suspension was kept to check for presence of HIV proviral DNA. The cells were washed three times, resuspended in MDDC culture medium and incubated for a further 5 days. The supernatant from the culture was collected and kept at -70°C for p24 ELISA and the cells were harvested for molecular analysis.

II.7. Statistical Analysis

Statistical analyses of allogeneic MLR and flow cytometry data were performed using SPSS 10.0 software. Statistical method employed was the 'independent-sample T test' to comparing two groups (e.g. control vs. 0.1%

seminal plasma-pulsed MDDCs, control vs. HIV-1 infected MDDCs). The equality of variance was assessed by 'Levene's test'. If the P values were less than 0.05 (i.e. 95% confidence) the results were regarded as significant. Data from allogeneic MLRs are presented in the form of stimulation indices (SI) with standard errors (SE) unless stated, which were evaluated using following equation:

$$SI = \frac{CPM_1}{CPM_2} \quad \text{..... (1)}$$

P values comparing the general trend curves presented in allogenic MLR data were determined based on the slope of each curve which was calculated using a Excel 97 software. General trend curves showing statistical significance are only presented in the appropriate figures. Data from flow cytometric analyses were presented in the form of mean fluorescence intensity (MFI) with standard errors and in the form of percentages of positive cells with standard errors. The MFI of each marker was determined by subtracting MFI of isotype matched control antibodies from the MFI of specific antibodies. The percentages of positive cells were determined by comparison with cells incubated with isotype matched control antibodies. Positive markers were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker. The degree of increase or decrease in levels of expression (or % positive cells) was calculated using following equation:

$$\frac{\Delta MFI \text{ (or \% positive cells)}}{} \times 100 \quad \text{..... (2)}$$

MFI_1 (or % positive cells)

(Where ΔMFI (or % positive cells) is difference in mean fluorescence intensities (or % positive cells) between control and treated MDDCs; MFI_1 (or % positive cells) is MFI (or % positive cells) of control MDDCs.

II.8. REAGENTS AND SUPPLIERS

Acetone	Merck
Agarose	Sigma
Antibodies	Refer Table II.1.
Anti-p24 antibody (D7320)	NIBSC
Biotinylated anti-p24 antibody	NIBSC
Brefeldin A (BFA)	Sigma
Bromophenol blue	Sigma
C-18 reverse phase column	SepPak, Waters
CD14 MicroBeads	Milltenyi Biotec
Chloroform	Sigma
Diemethyl sulphoxide (DMSO)	Sigma
DNA ladder marker	Gibco-BRL
Earls balanced salt (EBS) medium	Sigma
Empigen	Calbiochem
Ethanol	Merck

Ethanolamine	Sigma
Ethidium bromide	Sigma
Ethylenediamine tetraacetic acid (EDTA)	Sigma
FACS permeablising solution	Becton Dickinson
Foetal calf serum (FCS)	Sigma
Glycerol	Sigma
GM-CSF (Lucomax $1.11x10^7$ units/mg)	Norvatis
[^{3}H]-thymidine	Amersham Pharmacia Biotec
Histopaque	Sigma
Ionomycin	Sigma
Isopropanol	Sigma
Lactic acid	Sigma
Lamb serum	Gibco-BRL
Magnesium Chloride ($MgCl_2$)	Sigma
Medicol Medium Isotonic	Medi-Cult
Nuclease-free water	Biogenesis
NucliSens HIV-1 RNA assay kit	Organon Teknika
Oligo $d(T)_{16}$	Perkin Elmer
P24 antibody (D7320)	NIBSC
PCR reagents	Perkin Elmer
(Buffer, $MgCl_2$, dNTP, Ampli-*Taq* DNA polymerase)	
pNPP phosphatase substrate	Sigma
Phosphate buffered saline (PBS)	Sigma

Phorbol 12-Myristate 13-Acetate (PMA)	Sigma
Phytohaemaglutinin (PHA)	Sigma
Primers	Perkin Elmer/Gibco-BRL
Pyruvic acid	Sigma
Recombinant IL-2 (rIL-2)	Sigma
Recombinant IL-4 (rIL-4)	Sigma
Prostaglandin E	Calbiochem
19-OH prostaglandin E2	Cayman
Reverse transcriptase (MuLV)	Perkin Elmer
RNAzol™ B	Biogenesis
RNase inhibitor	Perkin Elmer
RPMI 1640 medium	Sigma
RQ1-RNase-free DNase	Promega
Sodium Azide (NaN_3)	Sigma
Sodium bicarbonate ($NaHCO_3$)	Sigma
Sodium chloride (NaCl)	Sigma
Sodium citrate	Sigma
Streptavidin-AP	Boehringer Mannheim
TNF-α	Sigma
Tri reagent™	Sigma
Tri-Borate EDTA (TBE)	Sigma
Trizma	Sigma
Trypan blue	Sigma

Tween 20	Sigma

Supplier addresses:

Amesham Pharmacia Boitech	Cambridge, U.K.
Becton Dickinson	Oxford, U.K.
Biogenesis	Poole, Dorset, U.K.
Boehringer Mannheim	Lewes, East Sussex, U.K.
Cayman	Alexis Corporation-Europe, Bingham, Nottingham, U.K.
Calbiochem	CN Biosciences (UK) Ltd, Beeston, Nottingham, U.K.
Gibco-BRL, Life Technoligy	Paisley, U.K.
Medi-Cult	Surrey, U.K.
Merck	Luttrworth, U.K.
Milltenyi Biotec	Bisley, Surrey, U.K.
NIBSC	Potters Bar, Hertfordshire, U.K.
Novartis	Farnborough, Hampshire, U.K.
Organon Teknika	Cambridge, U.K.
Perkin Elmer	Warrington, Cheshire, U.K.
Promega	Southampton, U.K.
SepPak, Waters	Watford, Hertfordshire, U.K.
Sigma	Poole, Dorset, U.K.

III

EVALUATION OF SPERM-WASHING AS A POTENTIAL METHOD OF REDUCING HIV TRANSMISSION IN HIV-DISCORDANT COUPLES WISHING TO HAVE CHILDREN

III.1. INTRODUCTION

Semen is an important vehicle in sexual transmission of HIV-1 and thus the level of virus in semen may be an important factor affecting the rate of transmission. HIV-1 is present in semen both as cell-free virus (Vernazza *et al*, 1996; Liuzzi *et al*, 1995; Liuzzi *et al*, 1996; Menzo *et al*, 1992; Gupta *et al*, 1997; Baccetti *et al*, 1991; Bagasra *et al*, 1988; Bagasrra *et al*, 1990; Rasheed *et al*, 1995; Coombs *et al*, 1998; Dyer *et al*, 1996) and non-sperm cell-associated virus (Vernazza *et al*, 1996; Baccetti *et al*, 1991; Bagasra *et al*, 1988; Bagasra *et al*, 1990), in particular associated with T lymphocytes and macrophages (Quyale *et al*, 1997). However, whether spermatozoa are infected with HIV remains controversial. A number of groups have presented data suggesting that HIV attaches to, and infects, spermatozoa (Bagasra *et al*, 1988; Bagasra *et al*, 1994; Baccetti *et al*, 1994; Scofield, 1992; Scofield *et al*, 1994; Gobert *et al*, 1990), possibly via CD4-like molecules (Ashida and Scofield, 1987; Bagasra *et al*, 1994; Socfield *et al*, 1992;

Scofield *et al*, 1994; Gobert *et al*, 1990), however some groups have failed to detect CD4 expression on sperm (Wolff and Anderson, 1988[a]; Wolff and Anderson, 1988[b]; El-Demiry *et al*, 1986). If CD4 or CD4-like molecules are present on sperm they could play an important role in infection of sperm with HIV-1 since CD4 is the primary cell receptor for HIV-1. Whilst some laboratories have provided evidence that spermatozoa are infected with HIV (Bagasra *et al*, 1988; Bagasra *et al*, 1994; Baccetti *et al*, 1991; Baccetti *et al*, 1994 Scofield *et al*, 1994) others could not detect HIV-1 particles or proviral sequences in spermatozoa (Mermin *et al*, 1991; Van Voorhis *et al*, 1991; Quayle *et al*, 1997).

There are a number of discordant couples, in whom the male is HIV positive and the woman HIV negative, who wish to have children. To conceive, they must abandon condom-protected intercourse risking HIV transmission to the woman and subsequently to her child. Semprini *et al* have conducted a program of assisted conception for such discordant couples since 1989 to minimise the risk of HIV transmission (Semprini *et al*, 1992). The assumption in this program was that spermatozoa are a reservoir for HIV in semen and therefore the spermatozoa are washed to remove seminal plasma and non-sperm cells (NSCs) by gradient centrifugation and 'swim-up' procedures, before use in artificial insemination. Such 'sperm washing' may reduce the amount of virus present in the spermatozoa sample, or even eliminate it completely, but this has previously only been evaluated by the relatively insensitive method of antibody detection of viral antigen on the processed sperm (Semprini *et al*, 1992). Artificial insemination timed with ovulation may also minimise the number of exposures of the

uninfected female partner to potentially infected material. To date Semprini's group have already carried out 1,690 inseminations for 543 couples resulting in almost 240 pregnancies with no seroconversion in mothers or their babies (Semprini, personal communication, 1999). Also, Marina *et al* reported that 101 inseminations using washed spermatozoa resulted in 31 pregnancies and both 28 mothers (3 miscarriages) and their 37 babies remained HIV seronegative (Marina *et al*, 1998). The aim of this part of thesis was to determine where HIV resides in semen. In particular we wished to determine whether HIV associates with spermatozoa, or are infected or have the potential to be infected by HIV and thus to evaluate whether 'sperm washing' is effective in risk reduction. A summary of methods used for 'sperm washing' and assays performed in this chapter are illustrated in fig.III.1.

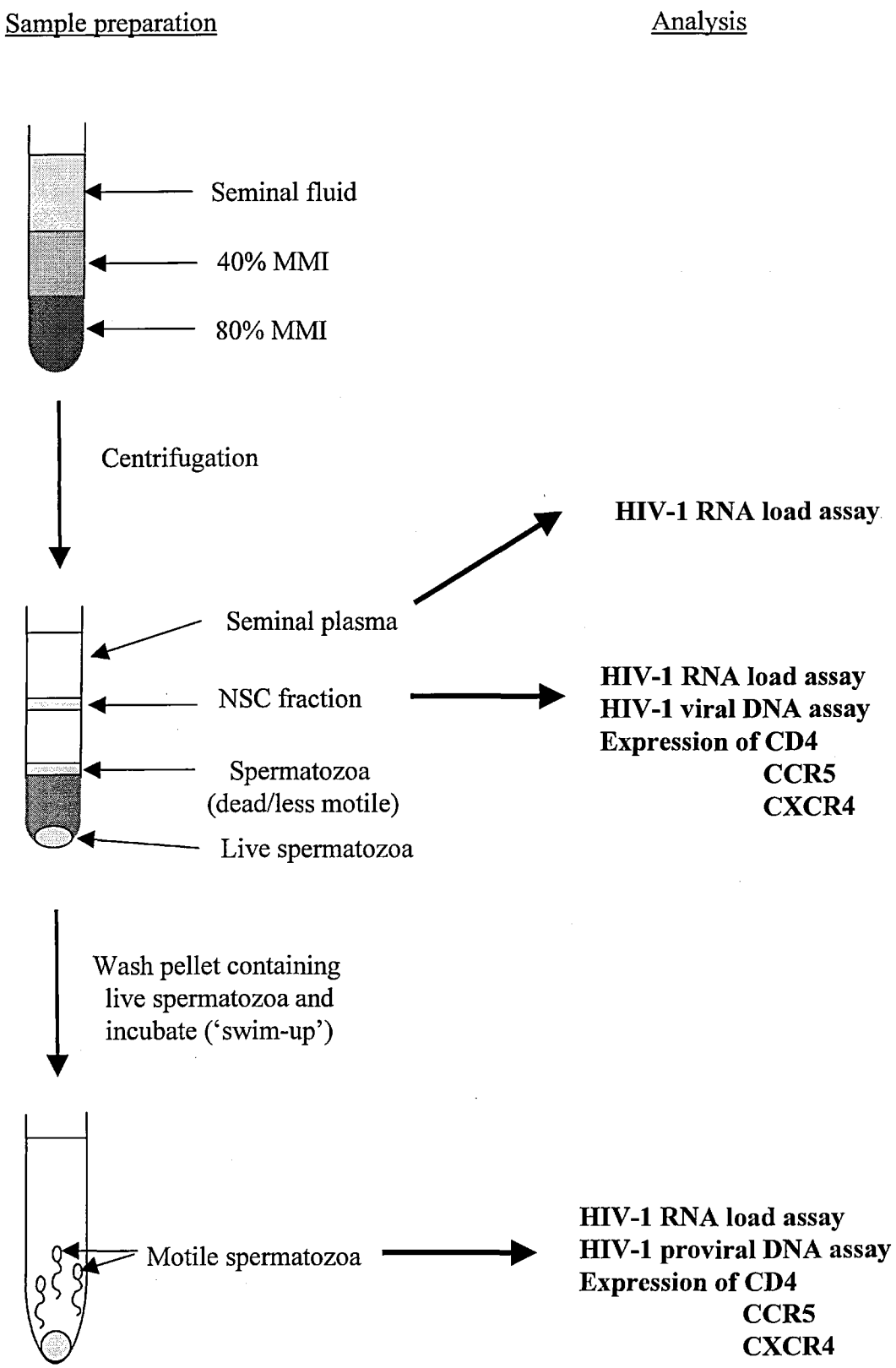
III.2. Results

III.2.1. HIV-1 RNA Load in Seminal Fractions and Blood Plasma

Blood and semen samples were obtained from eleven HIV-1 positive patients with blood CD4 counts of 93-786 cells/μl. Whole semen was separated into plasma, live 'swim-up' spermatozoa and NSC by differential gradient centrifugation followed by a 'swim-up' process (fig.III.1). The 'swim-up' procedure reduced the sperm count by up to one log compared with that in the whole ejaculate as reported by others (Lasheeb *et al*, 1997). The level of viral RNA in spermatozoa

Fig.III.1. Diagram showing semen sample preparation and analyses of each seminal component

Each semen sample was separated into seminal plasma, non-spermatozoa and motile spermatozoa by differential centrifugation over 40%/80% Medicol Medium Isotonic (MMI) followed by a 'swim-up' process. All three fractions were assessed for HIV-1 RNA load and presence of viral DNA. Expression of CD4, CCR5 and CXCR4 on non-sperm cells and spermatozoa were analysed by flow cytometry.


Sample preparation
Analysis
Seminal fluid
40% MMI
80% MMI
Centrifugation
HIV-1 RNA load assay
Seminal plasma
NSC fraction
HIV-1 RNA load assay
HIV-1 viral DNA assay
Expression of CD4
CCR5
CXCR4
Spermatozoa
(dead/less motile)
Live spermatozoa
Wash pellet containing
live spermatozoa and
incubate ('swim-up')
Motile spermatozoa
HIV-1 RNA load assay
HIV-1 proviral DNA assay
Expression of CD4
CCR5
CXCR4

was compared with that in whole semen and in blood plasma. Viral load assays were performed using a commercial assay, NucliSens™ which has previously been reported to be a more reliable assay for evaluation of viral load in semen samples compared with Amplicor (Roche) (Dyer *et al*, 1996). It has also been shown that semen has inhibitory activity against *Taq* DNA polymerase resulting in insufficient viral load assay (Dyer *et al*, 1996), and we demonstrated that inhibition could be avoided by extraction of RNA with silica as employed in the NucliSens™ system. The assay was performed on 100µl of blood plasma, 100µl of seminal plasma (diluted 1:4), 100µl unseparated semen (diluted 1:10), 1-10 x 10^5 spermatozoa, or 1-10 x 10^5 NSCs. Frozen and thawed blood plasma and seminal plasma samples were used. NSCs and spermatozoa fractions in lysis buffer were snap frozen prior to the assay (II.4.3. and II.5.3). The aim was to determine where HIV was located and also to examine whether 'sperm washing' significantly reduced the viral load in the spermatozoa to be used for insemination. Table III.1 illustrates that in all semen samples the 'swim up' procedure reduced the amount of virus in the spermatozoa to lower than the detection limit (LDL; ranged from 20 to 80 copies per ml per sample tested). HIV-1 RNA was not detected in the non-swim up spermatozoa or the 80%/40% interface (non-motile spermatozoa). The NucliSens™ assay incorporates three standard calibrators into each sample before RNA extraction, which demonstrates efficient RNA extraction and amplification for every sample tested. The calibrators amplified with the same efficiency in all samples tested (with the exception of patient 6 in the spermatozoal fraction) and therefore there was no evidence to suggest that there was any variation in the extraction of RNA or

amplification between fractions tested. No evidence that spermatozoa, even when dead or non-motile, were reservoirs of HIV RNA, was found. Four out of eleven samples were positive for HIV RNA in the NSC fraction, all of whom had significant viral loads in the whole semen sample. The data in Table III.1 indicates that the viral reservoirs in semen are the seminal plasma and the NSCs.

III.2.2. Proviral DNA Detection in Seminal Cells and PBMCs

It was possible that washed spermatozoa may contain latent virus in the form of integrated proviral DNA. Therefore each of the seminal fluid cellular fractions and PBMCs were tested for HIV-1 DNA by nested PCR (II.5.2) using primers designed to specifically amplify a highly conserved region of the HIV-1 envelope region and primers for β-globin as a positive control. DNA from PBMCs (10^6 cells) was extracted using Tri Reagent™ (II.4.1). Only a small number of NSCs (between $3x10^5$ and $5x10^6$ total) were recovered and therefore initially Tri Reagent™ was used as this allows extraction of both DNA and RNA from same sample. However, this method was not efficient in extracting DNA and also the nucleic acid extracted was not amplified by PCR due to PCR inhibitory factors in semen (Dyer *et al*, 1996) as mentioned above. Thus extracting DNA by the silica capturing method of the NucliSens™ (II.4.3) was used as an alternative when extracting DNA from semen, spermatozoa and NSCs. We have also found that spermatozoa were not easily lysed using Tri Reagent™.

Patient Number	Date of Diagnosis	Treatment	CD4 (No. cells μl^{-1})	Blood Plasma (Copies ml^{-1})	Seminal Fluid (Copies ml^{-1})	Seminal Plasma (Copies ml^{-1})	NSC Copies (no. cells)	Spermatozoa Copies (no. cells)
1	1. 8. 91	d4T, 3TC, Ritonavir, Saquinavir	425	150	LDL	LDL	LDL (10^6)	LDL (10^6)
2	1. 1. 86	d4T, 3TC, Saquinavir	786	LDL	LDL	LDL	LDL ($5x10^5$)	LDL ($6x10^5$)
3	1. 1. 86	Nil	187	8,300	LDL	LDL	LDL (10^6)	LDL ($1x10^6$)
4	1. 3. 95	AZT, 3TC, Indinavir	279	LDL	LDL	LDL	LDL ($5x10^5$)	LDL ($5x10^5$)
5	1. 1. 93	ddI, 3TC, Nelfinavir, Nevaripine	131	380,000	LDL	7,600	LDL ($5 x10^5$)	LDL ($4 x10^5$)
6	21. 3. 89	d4T, 3TC, Nelfinavir	369	1,500	5,000	LDL	LDL (10^6)	Invalid
7	1. 1. 94	Nil	93	400,000	11,000	5,200	1,700 ($3x10^5$)	LDL (10^5)
8	1. 8. 89	AZT, ddI	364	8,600	21,200	2,080	780 ($5x10^5$)	LDL ($6x10^5$)
9	15. 1. 92	d4T, 3TC, DMP266	187	1,200	56,000	2,000	24,000 ($5x10^6$)	LDL ($5x10^6$)
10	9. 8. 94	ddI, d4T, Nevaripine	247	69,000	176,000	68,000	54,000 (10^6)	LDL (10^6)
11	6. 6. 94	ddI	281	200,000	200,000	68,000	LDL (10^6)	LDL ($6x10^5$)

Table III.1. Viral Load in Semen and Blood

Whole semen was separated into seminal plasma and its cellular components [non-sperm cells (NSC) and spermatozoa] and the level of viral RNA determined by NucliSens™. All samples were successfully amplified except the spermatozoal fraction for patient 6, which inhibited the amplification in two tests. A number of samples were tested on more than one occasion resulting in comparable values.

The sensitivity of the DNA PCR was tested by isolating and amplification of DNA from a defined number of OM10.1 cells (NIBSC); each cell carries a single copy of HIV proviral DNA. By logarithmic end point dilution of the DNA and PCR using β-globin and the HIV *env*-primers it was demonstrated that these products were amplified with the same efficiency, and that the PCR could detect an input of 1 to 10 β-globin or HIV *env* sequences i.e. 1-10 cells (fig.III.2). A negative result was concluded only if the sample was negative in three separate experiments. Ten of the eleven patients had viral DNA present in PBMCs, unfractionated semen and NSCs. Patient 4 did not have detectable viral DNA present in PBMCs or NSCs although the unfractionated semen sample was positive in two out of three reactions (fig.III.3). Patient 4 also did not have detectable viral RNA in any of the compartments studied (Table III.1). Viral DNA was undetected in all spermatozoa samples analysed (Fig.III.3).

III.2.3. Expression of CD4 and HIV-1 co-receptors on seminal Cells

The potential of the spermatozoa to be infected by HIV-1 or the possibility that virus could simply attach to the spermatozoa cell surface was determined by evaluating expression of CD4 or the HIV co-receptors CCR5 or CXCR4 by flow cytometry in these cells (II.3.1). The data illustrated in Fig.III.4 are representative (sample 1) of the profiles obtained from 5 healthy volunteers. The percentages of positive cells were determined by comparison with cells incubated with isotype matched control antibodies. Positive markers (M1) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker.

Fig.III.2. Nested PCR showing sensitivity of viral DNA detection

OM10.1 cells which contain one copy of HIV-1 viral DNA were used to test the sensitivity of the PCR. The β-globin gene is a house-keeping gene (ie. one copy per cell) and was used as a positive control. The amount of DNA used in this reaction was equivalent to $7x10^3$ cells [Neat (N)] and the DNA was diluted (10^{-1} to 10^{-7}) to reach the end-point (ie. no DNA). 50 bp marker (M) was used. The data demonstrates that the PCR assay can detect viral and β-globin DNA with as few as 1-10 copies.

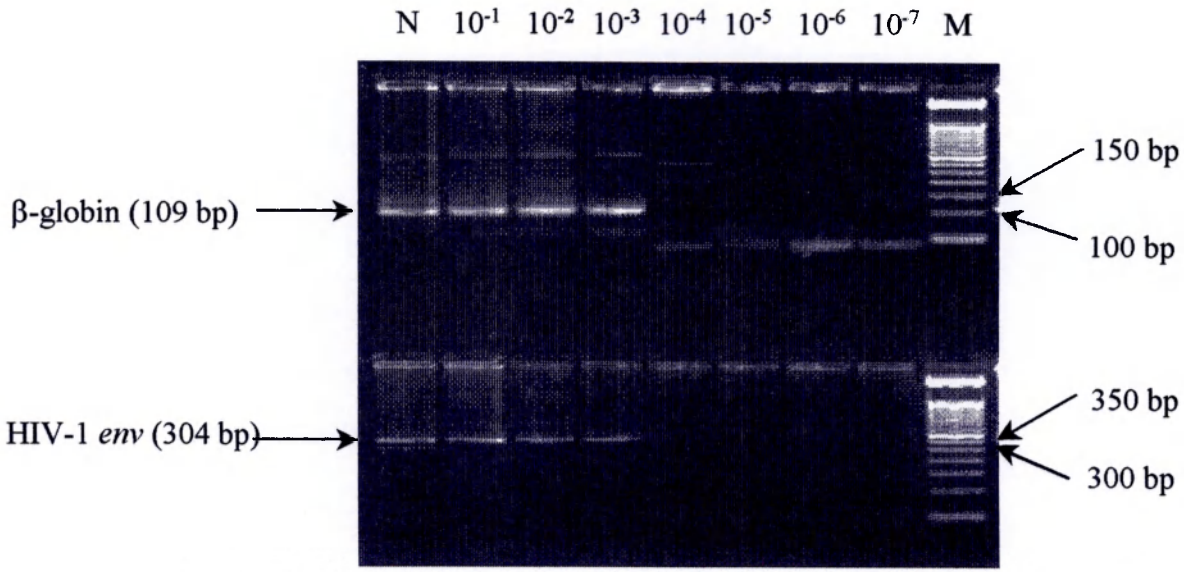
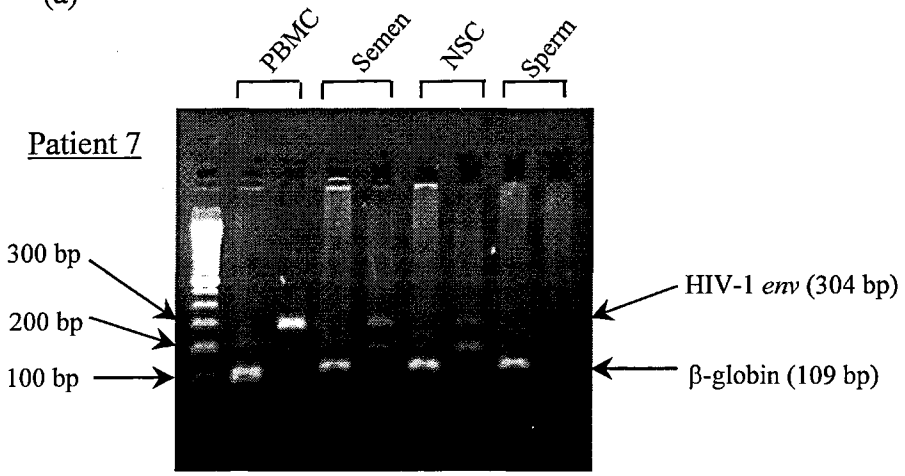


Fig.III.3. Viral DNA detection by Nested PCR

Viral DNA in peripheral mononuclear cells and seminal cellular components were assessed by a nested PCR and visualized on 1.5% agarose gel in the presence of ethidium bromide. The representative gel shown is patient 7 (a). The assay result from patient 4 shows that proviral DNA was detected only in unfractionated semen (b). Lane 1 represents β-globin gene amplification (product size 109 bp). Lane 2 represents HIV-1 envelope gene amplification (product size 304 bp).

(a)



	M	1	2	1	2	1	2	1	2
Patient 1	+	+	+	+	+	+	+	+	−
Patient 2	+	+	+	−	+	−	+	−	
Patient 3	+	+	+	+	+	+	+	−	
Patient 4	+	−	+	+	+	−	+	−	
Patient 5	+	+	+	+	+	+	+	−	
Patient 6	+	+	+	+	+	+	+	−	
Patient 7	+	+	+	+	+	+	+	−	
Patient 8	+	+	+	+	+	+	+	−	
Patient 9	+	+	+	+	+	+	+	−	
Patient 10	+	+	+	+	+	+	+	−	
Patient 11	+	+	+	+	+	+	+	−	
		PBMC		Semen		NSC		Sperm	

(b)

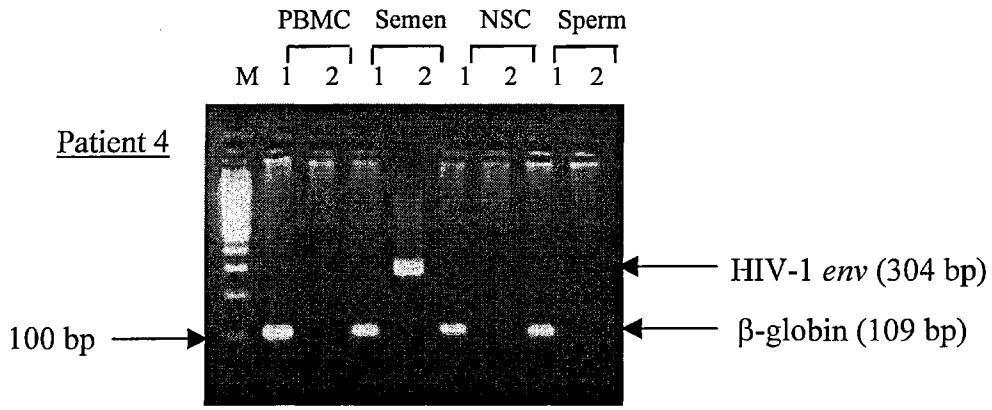
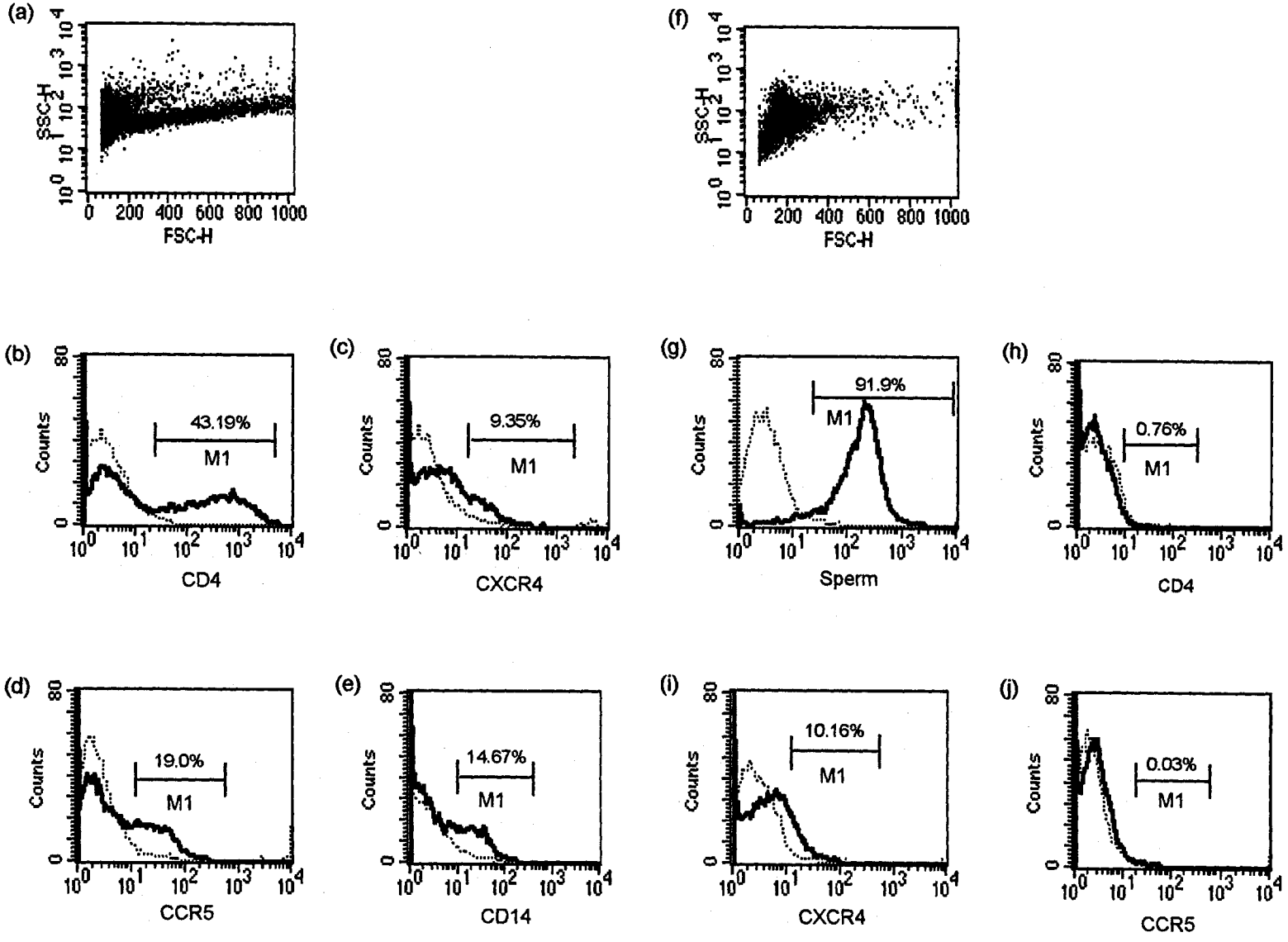


Fig.III.4. Expression of Cell Surface Markers on Seminal Cells

Semen was separated into non-spermatozoa cells (NSC) (a) and spermatozoa (f). NSC were stained with antibodies directed against the HIV receptors, CD4 (b), CXCR4 (c), CCR5 (d) and also the monocyte marker CD14 (e). The spermatozoa fraction was confirmed with anti-sperm antibody (g) and evaluated for expression of HIV receptors, CD4 (h), CXCR4 (i), and CCR5 (j). Dotted lines represent isotype controls and the solid lines represent specific antibody binding.


(a)
SSC-H
FSC-H
(f)
SSC-H
FSC-H
(b)
Counts
43.19%
M1
CD4
(c)
Counts
9.35%
M1
CXCR4
(g)
Counts
91.9%
M1
Sperm
(h)
Counts
0.76%
M1
CD4
(d)
Counts
19.0%
M1
CCR5
(e)
Counts
14.67%
M1
CD14
(i)
Counts
10.16%
M1
CXCR4
(j)
Counts
0.03%
M1
CCR5

The 'swim-up' procedure yielded very pure populations of spermatozoa cells (Fig.III.4g). Fig.III.4h and Fig.III.4j demonstrate absence of detectable CD4 and CCR5 on the spermatozoa cell surface. Two different clones of anti-CD4 antibodies were used (fig.III.4h for Q4120 and fig.III.5c for MT310). Low levels of CXCR4 expression were observed (Fig.III.4i) on spermatozoa. Whether this level of CXCR4 would be sufficient to allow X4 viruses to bind must be confirmed *in vitro*, although lack of expression of CD4 and CCR5 on spermatozoa suggests that these cells are very unlikely to be readily infected with R5 strains. Within the NSCs a population of cells exists with intense expression of CD4 (Fig.III.4b). These cells may be $CD3^+$ lymphocytes or monocytes as shown by expression of CD14 (Fig.III.4e). Within the NSC low levels of CXCR4 were detected compared with the isotype control (fig.III.4c), but there was a distinct population of CCR5 expressing cells (Fig.III.4d). This may suggest the possibility of compartmentalisation of CCR5 utilising viruses within semen which is discussed below. A summary of phenotypic analyses of seminal cells obtained from 5 heath individuals is illustrated in table III.2.

To determine whether seminal cells co-express both CD4 and CCR5 or CD4 and CXCR4, two colour staining was attempted but was not successful:

i) Staining cells with anti-CD4 antibody first, followed by washing and staining with anti-CCR5 antibody (fig.III.6a) or anti-CXCR4 antibody (fig.III.7a).

ii) Staining cells with anti-CCR5 antibody (fig.III.6b) or anti-CXCR4 antibody (fig.III.7b), followed by washing and staining with anti-CD4 antibody.

iii) Staining cells with anti-CD4 antibody and anti-CCR5 antibody (fig.III.6c) or anti-CXCR4 antibody (fig.III.7c) together at the same time.

As shown in fig.III.6 and III.7 the cells were only stained with first antibody or did not stain at all when stained as in (iii). This could be due to the fact that CD4 and HIV-1 co-receptors are expressed close to each other on the cell surface ('steric hindrance') (Xiao *et al*, 1999; Lapham *et al*, 1999).

Table III.2. Summary of phenotypic analyses of seminal cells from HIV-1 negative individuals

(a) Percentages of NSCs expressing CD4, CCR5, CXCR4 or CD14.

	CD4	CCR5	CXCR4	CD14
Sample 1	43.2%	19.0%	9.4%	14.7%
Sample 2	44.0%	11.5%	6.2%	8.6%
Sample 3	24.8%	23.0%	5.8%	18.1%
Sample 4	38.8%	21.2%	7.8%	10.4%
Sample 5	41.3%	18.1%	8.9%	18.5%
Mean±SE	38.8±3.5	18.6±1.9	7.6±0.7	14.0±2.0

(a) Percentages of spermatozoa expressing CD4, CCR5 or CXCR4.

	CD4	CCR5	CXCR4
Sample 1	0.8%	0.03%	10.2%
Sample 2	1.0%	0.5%	7.5%
Sample 3	0.8%	0.5%	2.7%
Sample 4	0.6%	1.6%	7.5%
Sample 5	1.0%	0.5%	7.5%
Mean±SE	0.8±0.07	0.6±0.3	7.1±1.2

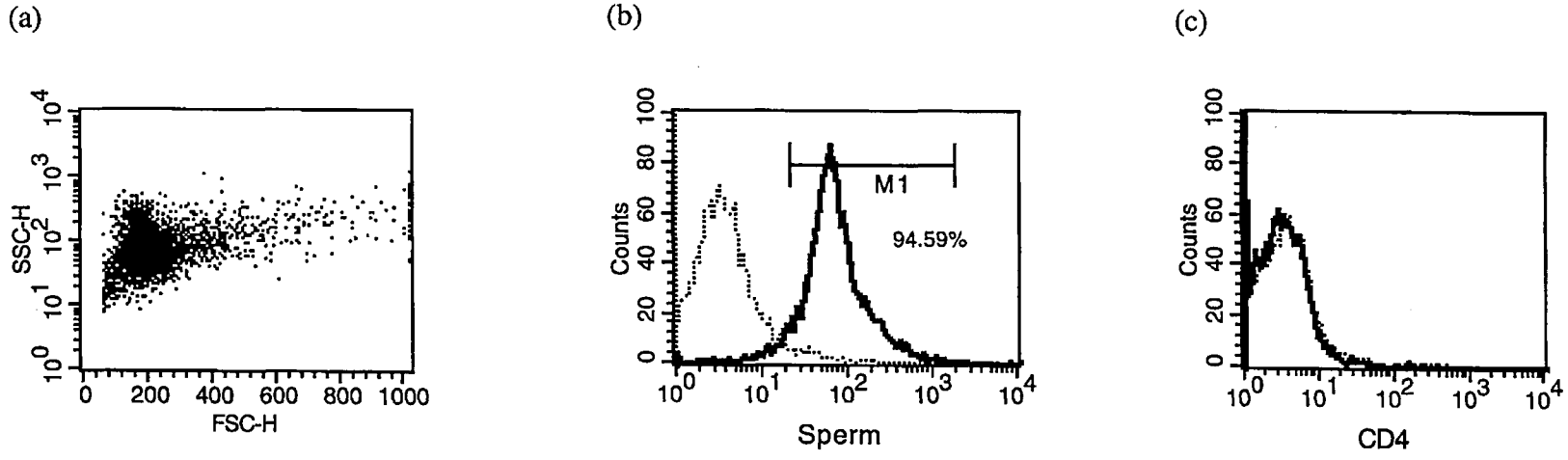


Fig.III.5. Detection of surface expression of CD4 on spermatozoa

(a) Dot plot showing spermatozoal fraction.

(b) Spermatozoa fraction was confirmed with specific binding of anti-sperm antibody.

(c) Using a different clone (MT310) of anti-CD4 antibody confirmed that spermatozoa do not express CD4 on the cell surface.

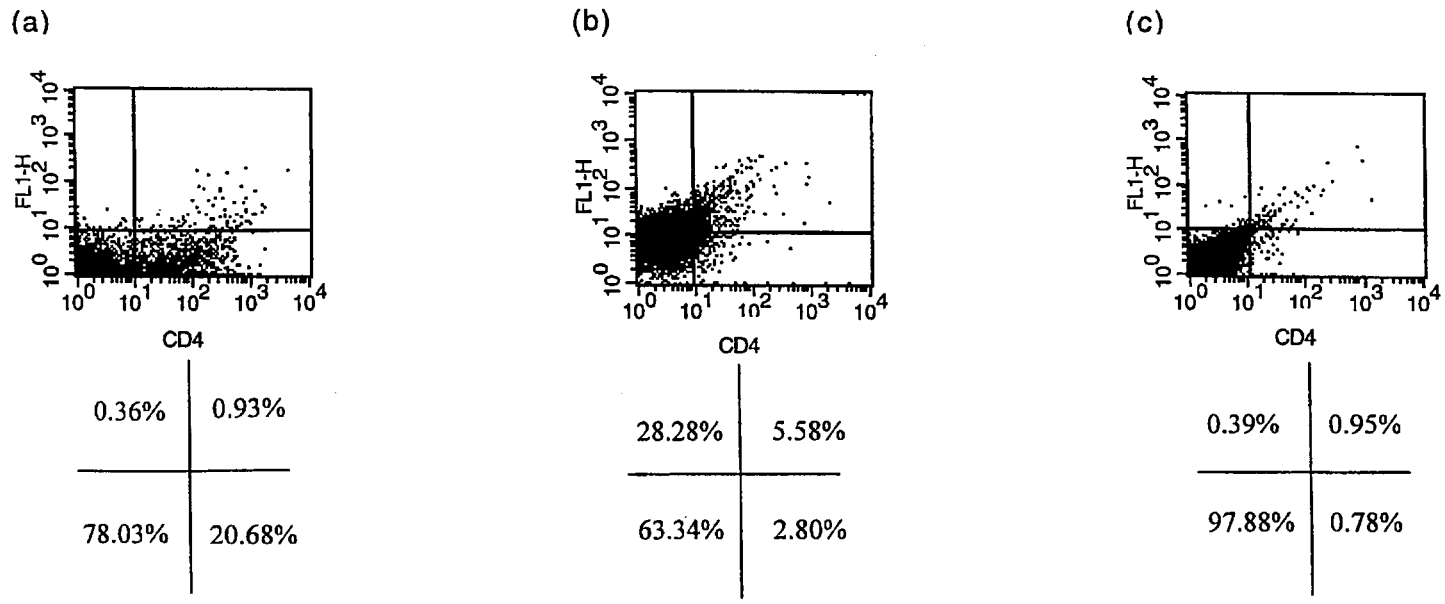


Fig.III.6. Double staining of seminal non-spermatozoal cells with anti-CD4 and anti-CCR5 antibodies (FL1=anti-CCR5 antibody)

(a) Staining with anti-CD4 antibody first followed by washing and staining with anti-CCR5 antibody. Cells were stained with anti-CD4 antibody only.

(b) Staining with anti-CCR5 antibody first followed by washing and staining with anti-CD4 antibody. Cells were stained with anti-CCR5 antibody only.

(c) Staining with both anti-CD4 antibody and anti-CCR5 antibody together at the same time. Most cells (97.88%) were not stained with either of antibodies.

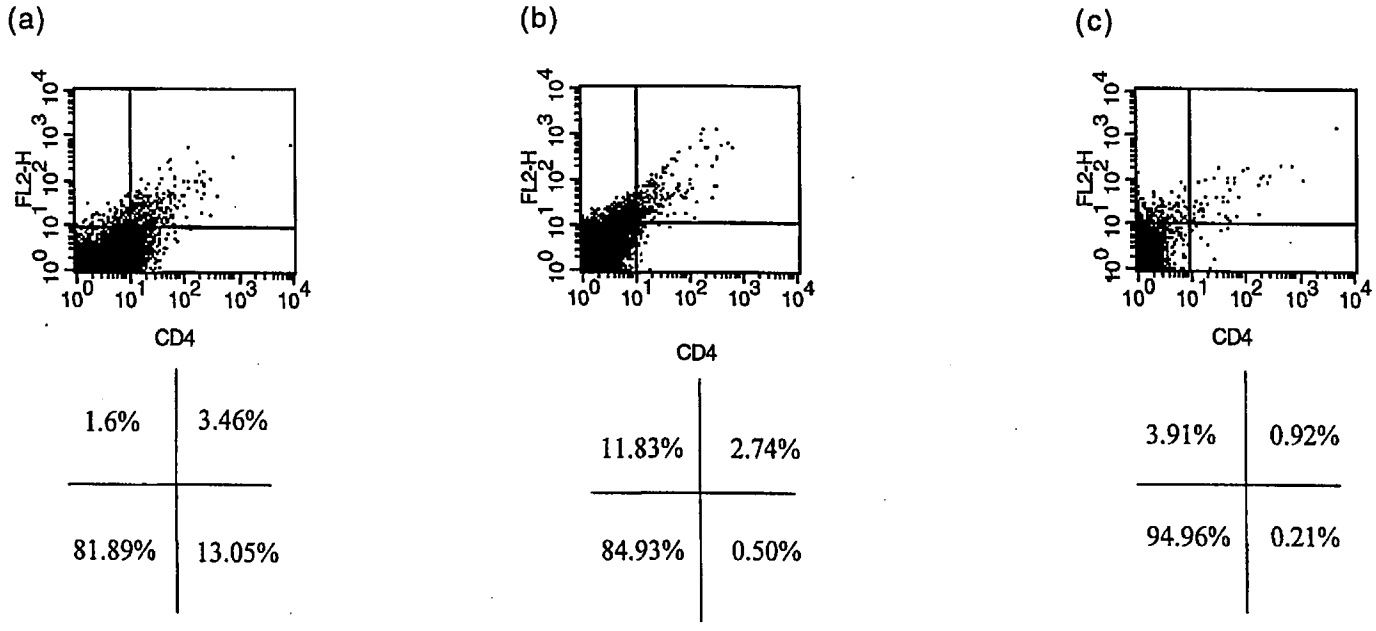


Fig.III.7. Double staining of seminal non-spermatozoal cells with anti-CD4 and anti-CXCR4 antibodies (FL2=anti-CXCR4 antibody)

(a) Staining with anti-CD4 antibody first followed by washing and staining with anti-CXCR4 antibody. (b) Staining with anti-CXCR4 antibody first followed by washing and staining with anti-CD4 antibody.

(c) Staining with both anti-CD4 antibody and anti-CXCR4 antibody together at the same time.

Percentage of cells stained with anti-CXCR4 antibody increased from 5.1% in (a) to 14.6% in (b). Percentage of cells stained with anti-CD4 antibody decreased from 16.5% in (a) to 3.2% in (b). About 95% of cells were not stained with either of the antibodies in (c).

III.2.4. RT-PCR for CD4

Expression of CD4 on spermatozoa was undetectable by flow cytometry (fig.III.4h). To confirm the data obtained by flow cytometry, expression of CD4 was also examined at the mRNA level by RT-PCR (II.5.1) using β-actin primers as a positive control. RNA from PBMC (10^6 cells) was extracted using Tri reagent™ (II.4.1.1.) and from spermatozoa using NucliSens™ Lysis buffer. Again, extraction of RNA from the seminal cellular fraction was attempted using Tri reagent™ and RNAzol™ B (II.4.2) but failed. Spermatozoa did not appear to be lysed using Tri reagent™ and RNAzol™ B. The results demonstrated that CD4 mRNA was not detectable in spermatozoa (fig.III.8) thus confirming the flow cytometry data.

III.2.5. Establishing a clinical service of 'sperm-washing' for HIV-discordant couples

Together with the Assisted Conception Unit, the Obstetrics and Gynaecology department and the HIV medicine department at Chelsea & Westminster Hospital we now provide a 'sperm-washing' service to HIV discordant couples. Patients undertake a series of counselling sessions and sign a consent form indicating that they understand that they are participating in a risk reduction, not a risk-free programme. Prior to receiving the 'sperm-washing' service both male and female partners undertake sexual health and gynaecology screens (table III.3).

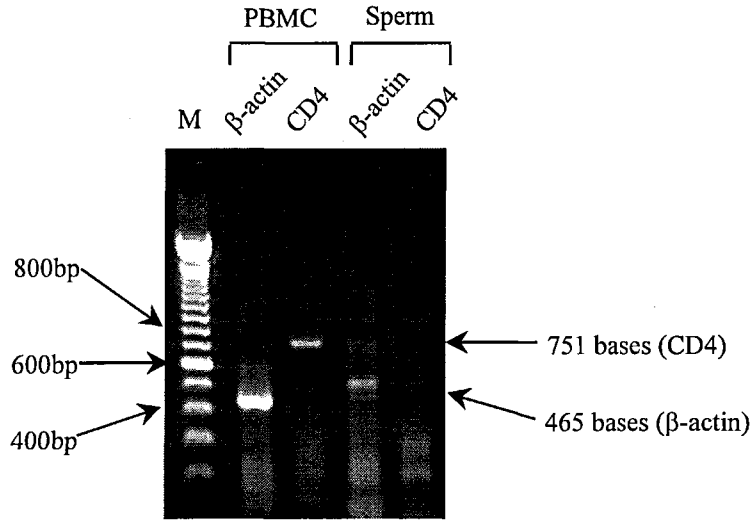


Fig. III.8. RT-PCR for CD4 mRNA from PBMC and spermatozoa

RNA was extracted from PBMC (positive control RNA) using Tri reagent and from spermatozoa using NucliSens Lysis buffer. cDNA was synthesised using oligo $d(T)_{16}$ and MuLV reverse transcriptase followed by PCR for β-actin as a positive control and CD4. The RT-PCR products were visualised on 1.5% agarose gel in the presence of ethidium bromide with 100bp marker (M).

Table III.3. Investigations required prior to intra-uterine insemination (IUI)

Sexual health screen		Baseline gynaecology tests	
Male	Female	Male	Female
Gonorrhoea Syphilis Chlamydia	HIV-1, -2 antibody Hepatitis B Hepatitis C Candida Gonorrhoea Trichomoniasis Syphilis Chlamydia Bacterial Vaginosis	Semen Analysis* (table III.4)	Full blood count Rubella Prolactin Thyroid function Follicle stimulating hormone Luteinising hormone E2 or Oestradiol Baseline ultrasound scan Ovulation check Hysterosalpingogram

*Criteria for continuing treatment: No less than $20x10^6$ spermatozoa, no more than 80% abnormal spermatozoa, and not less than 50% motility.

Semen sample preparation and analysis are carried out in the Immunology department. *In vitro*-fertilisation (IVF) medium is incubated at 37°C, 5% CO_2 overnight prior to semen preparation. The semen samples are processed within one hour of donation as described in II.3.2, except that Medicol Medilum Isotonic (MMI) was replaced by PureSperm® as MMI is commercially no longer available. Semen parameters are initially assessed as outlined by the World Health Organisation (table III.4).

Table III.4. Normal semen parameters outlined by WHO

Volume	>2.0ml
pH	7.2-8.0
Sperm concentration	$>20x10^6$/ml
Total sperm count	$>100x10^6$
Motility	>50% with forward progression or 25% or more with rapid progression within 60 minutes of ejaculation
Morphology	>30% with normal forms
Vitality	>75% live
White blood cell count	$<2x10^6$/ml
Immunobead test	<20% spermatozoa with adherent particles
MAR test (test for presence of IgG on the spermatozoa)	<10% spermatozoa with adherent particles

A fraction of the purified spermatozoa cells to be used for the HIV-1 RNA test (10^6 cells) is snap frozen and thawed quickly prior to the assay to ensure complete cell lysis since we found that spermatozoa cells are not readily lysed by other methods. HIV-1 RNA levels in the purified spermatozoa cell fraction are quantitated using NucliSens™ (10^6 cells per test). The woman is inseminated with 'washed' sperm only when HIV-1 RNA load in the tested fraction of washed

sperm is below the detection limit. Each patient may undergo three insemination attempts and if the woman fails to become pregnant after three attempts, the couple may be advised to receive *in vitro* fertilisation with washed sperm. Table III.5 illustrates semen and purified spermatozoa analyses. The motility of spermatozoa after 'Sperm-washing' procedure was between 85% and 100%. In some patients, motility of sperm cells and total sperm counts in whole semen were lower compared with normal semen parameters outlined by WHO (table III.4). This may be because HIV interferes with spermatogenesis. Arrested spermatogenesis in the testes in AIDS has been reported (Muciaccia *et al*, 1998). It also has been shown that TNF-α, which has been shown to be increased in blood plasma of HIV patients (Lore *et al*, 1999), has negative effects on sperm motility (Estrada *et al*, 1997). Spermatogonia express galactosylceramides (Brogi *et al*, 1995) which is a component of the neural receptor for HIV (Bath *et al*, 1991; Harouse *et al*, 1991), suggesting that this glycolipid could be involved in a potential interaction of HIV with spermatogonia, but this hypothesis has not been confirmed. Following 63 inseminations in 31 women, 12 women have become pregnant. However, 5 miscarriages have occurred but there have been 3 live births with 4 currently ongoing. In addition 9 women have undergone IVF treatment resulting in 1 pregnancy. All these women have remained seronegative.

III.3. DISCUSSION

The viral reservoir in semen was investigated and furthermore, whether spermatozoa are infected or have potential to be infected with HIV was also determined. Whether spermatozoa are infected with HIV has been controversial. This is of particular importance in the evaluation of 'sperm washing' as a viable risk reduction option for HIV discordant couples wishing to have children. We did not demonstrate the presence of CD4 on the spermatozoa cell surface using two different clones of anti-CD4 antibodies and also mRNA for CD4 was not detected. There was no surface expression of CCR5 on spermatozoa. Although a low level of CXCR4 expression was observed it would be very unlikely that spermatozoa are susceptible to infection with HIV since we demonstrated clearly that there was no detectable surface expression of CD4 on spermatozoa cells or CD4 mRNA in spermatozoa cells. Neither HIV-1 RNA nor DNA was detected in any spermatozoa fraction of any of our subjects. Our data demonstrate that 'sperm washing' achieves substantial reductions in the level of virus present, although due to our sample size it is not possible to guarantee that the LDL would be achieved with all patients. Our data is in concordance with clinical data from Semprini and colleagues where no HIV infection has occurred after 1,690 inseminations with washed sperm (Semprini, 1999, Personal communication). Our data was further supported by recent studies led by Izopet (Pasquier *et al*, 2000) who demonstrated removal of HIV-1 after a 'sperm washing' process. A study by Lasheeb *et al* indicated that 'sperm washing' reduced HIV viral load in semen, although in this case the spermatozoa were separated after freezing of the

semen sample, which could result in cell lysis, allowing RNA contamination of the cellular compartments (Lasheeb *et al*, 1997). In our experiments semen was donated from patients with a range of viral loads in peripheral blood (LDL to 400, 000 copies ml^{-1}). Six individuals had HIV RNA present in the seminal plasma, four of whom had viral RNA present in the NSCs. Nine individuals had detectable proviral DNA in the NSC. The main viral reservoir in semen was the seminal plasma and NSC (Table III.1). The NSCs represent a heterogeneous population containing immature germ cells, leukocytes and epithelial cells. The leukocyte fraction contains $CD68^+$ macrophages, $CD3^+$ T cells (both $CD4^+$ and $CD8^+$) and $CD103^+$ T cells which are found in the epithelium and lamina propria (Quayle *et al*, 1997). The main reservoir for HIV appears to be the lymphocyte and macrophage populations, but not the immature germ cells (Quayle *et al*, 1997). Poor correlation was found between the viral load in semen and that in peripheral blood (Table III.1). For example patient 5 had 380,000 copies ml^{-1} in blood and LDL in semen, and patient 9 had only 1,200 copies per ml in blood but 56,000 copies ml^{-1} in unfractionated semen. Other groups report similar findings (Liuzzi *et al*, 1996; Coombs *et al*, 1998; Gupta *et al*. 1997). Shedding of virus into semen may be intermittent with the level of seminal virus increasing with factors such as decreased CD4 count and asymptomatic genital tract infection (Vernazza *et al*, 1997; Xu *et al*, 1997). Therefore because the presence of HIV in blood plasma is not always correlated with its presence in semen, detection of virus in blood plasma may not be a good surrogate for infectivity via sexual transmission. The data also suggests that blood and semen may represent two distinct compartments in terms of HIV replication. There is some evidence provided by

other groups (Rasheed *et al*, 1995; Liuzzi *et al*, 1996; Liuzzi *et al*, 1995; Gupta *et al*, 1997; Coombs *et al*, 1998; Zhu *et al*, 1996; Delwart *et al*, 1998; Kiessling *et al*, 1998; Byrn *et al*, 1997; Roos *et al*, 1992) as discussed in chapter I, suggesting that HIV in semen may not arise from the same reservoir as in peripheral blood.

It has been postulated that during sexual transmission of HIV the selection of the R5 viruses takes place at the mucosal surface, since it is the Langerhans cells (LCs) which may be the first cells to become infected (Spira *et al*, 1996; Blauvelt, 1997; Zambruno *et al*, 1995; Miller and Hu, 1999), and these cells express functional CCR5 but not CXCR4 (Zaitseva *et al*, 1997). It has been postulated that semen is a major reservoir for the CCR5 utilising viruses thus allowing preferential transmission of R5 strains. This hypothesis is supported by phenotypic and genotypic analyses of viral isolates from blood and semen (Zhu *et al*, 1996; Kroodsma *et al*, 1994) which found predominantly M-tropic virus present in semen regardless of the tropism of virus found in the periphery. We have shown that the NSCs express CCR5, but negligible amounts of CXCR4 suggesting that this may contribute to the selection of R5 viruses within semen. However, restriction of SI variants from the male genital tract is not always observed (Delwart *et al*, 1998). As sexual transmission accounts for the vast majority of HIV transmission it is of pivotal importance to determine whether the virus within semen represents a distinct compartment of viral replication and hence viral phenotype. An effective prophylactic vaccine must confer protection against the phenotype shed in genital secretions.

'Sperm washing' as a risk reduction program has been a controversial area. Mandelbrot *et al* (Mandelbrot *et al*, 1997) believe the risk of transmission is sufficiently low that HIV discordant couples may attempt natural conception. They report a follow up of 92 HIV negative women with HIV positive partners. Most couples had received pre-conceptual counselling on the risk of transmission, and were advised to pinpoint ovulation to reduce the risk. Two women seroconverted at seven months of pregnancy and another two post-partum, with seroconversions restricted to couples with inconsistent condom use. Perhaps advising individuals that they may abandon condoms to conceive may encourage intermittent condom use. Our data suggest that isolating the sperm for insemination could greatly reduce such a risk.

Antiretroviral therapy has a marked effect on HIV shedding in semen, as treatment-induced changes in viral load in blood are generally reflected by corresponding changes in viral load in semen (Vernazza *et al*, 1997). Thus a further risk reduction exercise might be to encourage all HIV^+ males who wish to participate in such a programme to consider commencing potent antiretroviral therapy, however the teratogenicity of many of the drugs currently in use has not been completely evaluated. Our data suggests that the primary reservoir for HIV RNA in semen is the seminal plasma and NSC, and that the level of viral RNA could be reduced to LDL in the washed spermatozoal fraction. Secondly all the spermatozoa fractions analysed for latent proviral DNA in this study were negative where the unfractionated semen (10 out of 11 patients) were positive. We have failed to detect the presence of CD4 or CCR5 on spermatozoa and the

expression of CXCR4 on the spermatozoa was low, suggesting that these cells are not likely to be readily infected by HIV. Therefore, we provide evidence to suggest that 'washing sperm' reduces the amount of HIV present and hence will reduce the risk of HIV transmission. Taken together with the clinical data from Semprini's group we would promote 'sperm washing' as an efficient and simple procedure to reduce the risk of HIV transmission in HIV discordant couples wishing to have children. Furthermore, it has been shown that the 'sperm-washing' process also reduced the risk of hepatitis C virus infection (Pasquier *et al*, 2000). We have tested for the presence of hepatitis C virus in purified spematozoa in one patient with known hepatitis C status and hepatitis C virus was undetected (data not shown). The 'sperm-washing' service is in the place within this hospital trust and we have performed 63 artificial inseminations in 31 women with 12 pregnancies and 3 live births.

Further work

i) Characterisation of the virus present in semen

Our data demonstrated that there is a distinct population of cells expressing CCR5 in NSC fraction. The tropism of the virus in semen could be determined.

ii) *In vitro* infection of LCs or LC-like DCs with viral isolates from semen

Whether viruses in semen can infect LCs or LC-like DC could be assessed (chapter V).

iii) *In vitro* infection of spermatozoa with X4 strains of HIV-1

Although we have demonstrated that spermatozoa do not express CD4 on the cell surface (Fig.III.3i) and do not produce mRNA for CD4 (fig.III.6) there was a low level of CXCR4 expressed on spermatozoa. To further analyse whether spermatozoa may be infected with HIV-1, *in vitro* infection of spermatozoa with virus could be performed.

iv) *In vitro* infection of NSCs with HIV-1

In vitro infection of NSCs with X4 strains of HIV-1 would allow assessment of whether the level of CXCR4 on NSC is in fact sufficient for infection with X4 viruses.

IV

IMMUNOMODULATORY EFFECTS OF SEMINAL PLASMA ON DENDRITIC CELLS

IV.1. INTRODUCTION

Dendritic cells (DCs) are bone marrow derived cells [except for the follicular DCs that are present within the B-lymphoid follicles of lymphoid tissue (Matsumoto *et al*, 1997)] and are the most potent antigen presenting cells (APCs). DCs initiate primary immune responses as they can take up, process and present antigens to and stimulate naïve T cells. Mature DCs are more than 100 times more potent than macrophages in activating naïve T cells *in vitro* (Banchereau and Steinman, 1998). DCs can also potentiate secondary immune responses by presentation to and stimulation of memory T cells. DCs at different maturation stages are found in both the non-lymphoid and the lymphoid tissues. DC progenitors migrate to non-lymphoid tissues, where they develop into 'immature' DCs. The 'immature' DCs have high capacity for capturing and processing antigen but poor T cell stimulatory capacity as they have little or no co-stimulatory activity (Austyn, 1998; Banchereaue and Steiman, 1998; Hart, 1997; Cella *et al*, 1997). 'Immature' DCs in tissues include Langerhans' cells (LCs) in the epidermis of skin and the mucosa. LCs take up antigen by macropinocytosis or by mannose receptor-mediated endocytosis (Sallusto *et al*, 1995). Pro-inflammatory cytokines such as TNF-α,

IL-1β (Sallusto and Lanzavecchia, 1994; Sallusto, *et al*, 1995) and IL-6 (Jonuleit, *et al*, 1997), and also the uptake of antigen, induce activation *in vitro*. The activated DCs migrate to the draining lymph nodes where they mature. Mature DCs have poor ability to capture and process antigen but express increased levels of cell-surface MHC class II and co-stimulatory molecules [e.g. CD80/B7.1, CD86/B7.2 and B7-DC (Tseng *et al*, 2001)]. Hence the mature DCs acquire the ability to present antigen acquired at the immature stage and can activate naïve T cells via a co-stimulatory.

Both semen and LCs in the mucosa may play an important role in transmission of sexually transmitted diseases (STDs). For example, in HIV infection via sexual contact, LCs in the lamina propria of the mucosa may be the first cells to become infected (Spira *et al*, 1996; Joag *et al*, 1997; Blauvelt *et al*, 1997; Zambruno *et al*, 1995; Miller and Hu, 1999) and semen is the vehicle for the virus. Seminal plasma also contains immunosuppressive components such as prostaglandins, prostasomes, polyamines and TGF-β (Alexander and Anderson. 1987; Kelly, 1995). It is known that prostaglandins which are present in human semen at about 10^8 fold higher concentrations than in peripheral blood (Kelly, 1997[a]), have immunomodulatory effects. For instance, prostaglandin E2 (PGE2) is a pro-inflammatory molecule and activates/matures DCs and subsequently induce IL-12 production by DCs (Riesser *et al*, 1997; Riesser *et al*, 1998; Portanova *et al*, 1996). PGE2, is also known to inhibit the production of pro-inflammatory cytokines by lipopolysaccharide (LPS)-activated macrophages (Strassmann *et al*, 1994). Thus, PGE2 inhibits LPS-induced IL-12 production by macrophages and

stimulates IL-10 production in whole blood culture (Kraan *et al*, 1995; Kelly *et al*, 1997[a]). This cytokine switch to Th2-type may induce anergy or peripheral tolerance. Taken together, PGE2 has both pro-inflammatory and suppressive activities for APCs. Prostaglandins in human seminal plasma include four main species which are 19-hydroxy PGE2, 19-hydroxy PGE1, PGE1 and PGE2. 19-OH PGE1 and 19-OH PGE2 are, as far as is known, unique to semen (reviewed by Kelly, 1997[b]). Cells in the mucosa of female reproductive tract do not recognise and respond to sperm or non-sperm cells in seminal fluid as foreign antigens, however, at the same time, they need to respond to potential pathogens in semen.

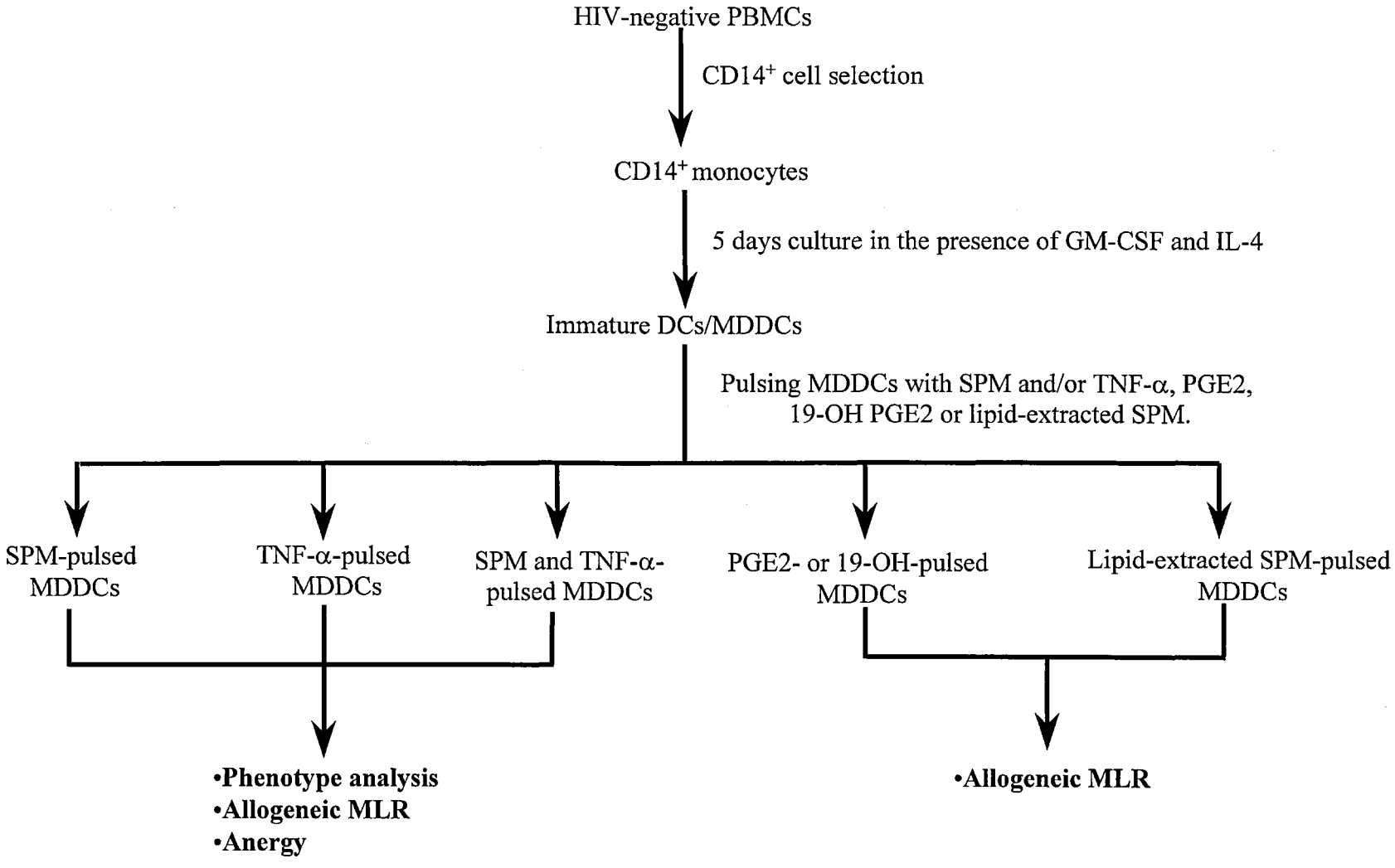
In this part of thesis a study of the effects of seminal plasma on the phenotype and function of DCs generated from $CD14^+$ cells (Sallusto and Lanzavecchia 1994), and the possible role of seminal plasma in transmission of STDs, including HIV, are assessed. A summary of these experiments is illustrated in fig.IV.1.

IV.2. Results

Data from allogeneic mixed lymphocyte reactions (MLRs) was presented in the form of mean counts per minute (CPM) with standard errors (SE) for triplicates and in the form of mean stimulation indices (SI) with SE, for single representatives and for summary of all experiments, respectively. Data from flow cytometric analyses was presented in the form of mean fluorescence intensity (MFI) with SE. The live MDDC population was gated and designated

Fig. IV.1. Flow chart describing experiments performed in chapter 4

$CD14^+$ monocytes were positively selected from PBMCs (HIV-negative) using CD14 MicroBeads (II.2.4). $CD14^+$ monocyte-derived DCs (MDDCs) were generated in the presence of GM-CSF and IL-4 (II.2.5). After 5 days of culture cells were pulsed with two different concentrations of seminal plasma (SPM) and/or 200U/ml of TNF-α (II.2.6). All MDDC populations were assessed for phenotypes (II.3.1) and allogeneic capacities (II.2.7). SPM-pulsed MDDCs were further assessed for anergy (II.2.8). MDDCs were also pulsed with PGE2, 19-OH PGE2 (II.2.6) or lipid-extracted SPM (II.2.2) after 5 days of culture and their allostimulatory abilities were analysed by allogeneic MLRs (II.2.7).

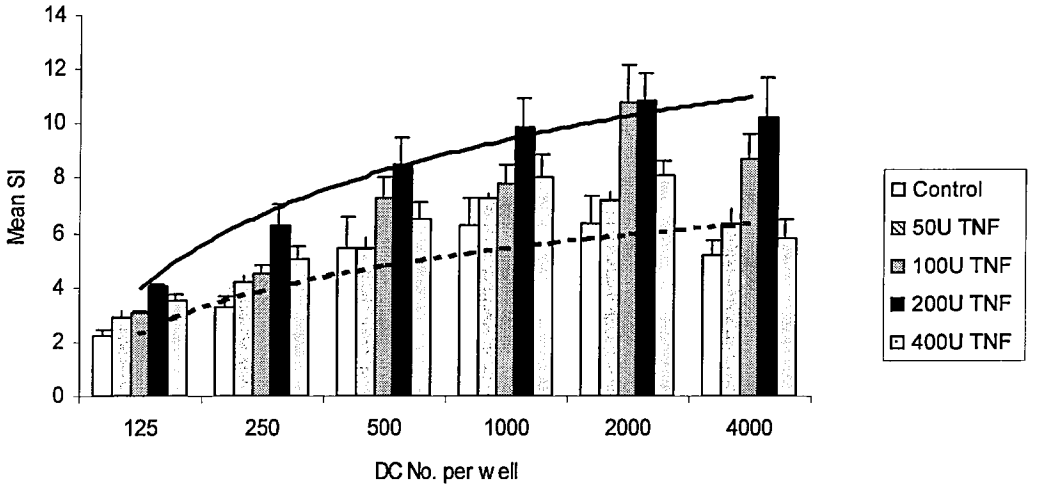

HIV-negative PBMCs
CD14+ cell selection
CD14+ monocytes
5 days culture in the presence of GM-CSF and IL-4
Immature DCs/MDDCs
Pulsing MDDCs with SPM and/or TNF-α, PGE2, 19-OH PGE2 or lipid-extracted SPM.
SPM-pulsed MDDCs
TNF-α-pulsed MDDCs
SPM and TNF-α-pulsed MDDCs
PGE2- or 19-OH-pulsed MDDCs
Lipid-extracted SPM-pulsed MDDCs
•Phenotype analysis
•Allogeneic MLR
•Anergy
•Allogeneic MLR

as R1 (fig.IV.4d, 5d, 6d) and expression of CD1a, CD4, HLA-DR, CD80 and CD86 on MDDCs was analysed within this population. The degree of increase or decrease in levels of expression was calculated using equation 2 described in II.7. The percentages of positive cells were determined by comparison with cells incubated with isotype matched control antibodies. Positive markers (M1 in fig.IV.4) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker. Single representatives of each flow cytometry profile are also shown.

IV.2.1. TNF-α and seminal plasma titration

Optimal concentrations of TNF-α and seminal plasma for use in this study and studies described in chapter 5 were determined. Fig.IV.2 shows the effect of different concentrations of TNF-α on the allostimulatory capacity of MDDCs. 200U/ml of TNF-α had the most potent effect on allostimulatory capacity of MDDCs compared with control MDDCs and hence 200U of TNF-α per ml of MDDC culture (10^6 cells/ml) was used in subsequent experiments.

MDDCs were pulsed with a pool of seminal plasma from six HIV-negative individuals as described in II.2.6. Overnight incubation of MDDCs with 10% seminal plasma resulted in a very low viable cell yield ['trypan blue exclusion' (II.2.1); less than 10^3 cells/ml] when examined under a light microscope. Furthermore, seminal plasma titration experiments demonstrated that cells pulsed with 10% seminal plasma failed to stimulate allogeneic T cells (fig.IV.3). This is maybe due to a toxic effect of seminal plasma resulting from



DC No. per well		125	250	500	1000	2000	4000	*General trend*
P values (vs. control)	50U TNF-α	0.2	0.4	1.0	0.6	0.7	0.4	*0.3*
	100U TNF- α	0.2	0.1	0.5	0.5	0.2	0.1	*0.06*
	200U TNF- α	**0.02**	0.1	0.3	0.2	**0.04**	0.09	***0.002***
	400U TNF- α	0.1	0.2	0.7	0.5	0.4	0.7	*0.1*

Fig. IV.2. TNF-α titration

MDDCs were pulsed with graded concentrations (50U/ml, 100U/ml, 200U/ml, 400U/ml) of TNF-α overnight after 5 days of cultivation. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. The graph shows a mean stimulation index (SI) with standard error for 3 experiments and each experiment was set up triplicate. The table below the graph shows P values comparing allostimulatory ability of TNF-α-pulsed DCs with that of control DCs. P value for the general trends of each curve (*italic*) are also shown.

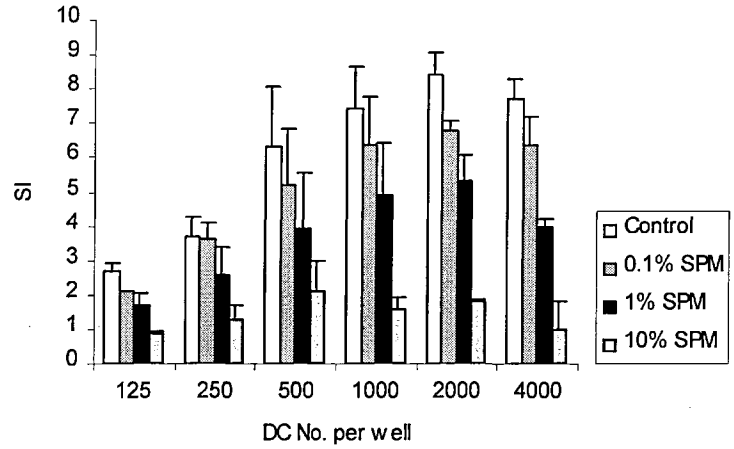


Fig.IV.3. Seminal plasma titration

MDDCs were pulsed with graded doses of seminal plasma (0.1%, 1%, 10%) overnight after 5 days of cultivation. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. The graph shows mean stimulation indices (SI) with standard errors for 2 experiments. Each experiment was set up in triplicate. Due to a small number of samples statistical analysis was not performed.

an interaction with bovine serum in MDDC culture medium, inducing the oxidative deamination of polyamines (spermine, spermidine and putrescine) in seminal plasma giving rise to formation of several toxic products such as aldehydes, ammonia and hydrogen peroxide (Agostinelli *et al*, 1994; Averill-Bates *et al*, 1993; Labib and Tomasi, 1981; Katsuta *et al*, 1975). Therefore, in subsequent experiments, a maximum of 1% seminal plasma was used to pulse MDDCs.

IV.2.2. Phenotypic characterisation of MDDCs treated with seminal plasma and/or TNF-α

The phenotype of MDDCs exposed to seminal plasma and/or TNF-α (as an example of a pro-inflammatory cytokine) was analysed by flow cytometry. The live MDDC population was gated and designated as R1 (fig.IV.4d, IV.5d, and IV.6d) and expression of CD1a, CD4, HLA-DR, B7.1/CD80 and B7.2/CD86 was analysed within this population. The percentages of positive cells were determined by comparison with cells incubated with isotype matched control antibodies. Positive markers (M1; fig.IV.4d, IV.5d, and IV.6d) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker.

Seminal plasma induced changes in expression of surface co-stimulatory molecules (fig.IV.4). MFI of CD80 expressed on $CD80^+$ MDDCs decreased significantly by 43.9%(±9.9) [i.e. from 31.2(±2.7) to 17.8(±4.3); ΔMFI=13.4(±2.9)] when MDDCs were pulsed with 1% seminal plasma

compared with that of control MDDCs (P=0.04; fig.IV.4b). However, such a decrease in the level of CD80 expression on MDDCs by 1% seminal plasma did not result in a complete disappearance of the molecule as demonstrated by the observation that numbers of CD80 positive MDDCs remained unchanged (P=0.8; fig.IV.4c). The MFI of CD86 expression on the total MDDC population was decreased significantly by 55.0%(±8.0) with 1% seminal plasma (P=0.04; fig.IV.4a): a decrease from 331.0(±67.5) to 144.2(±29.5) [ΔMFI=186.8(±51.1)]. This reduction in the level of CD86 expression in the total MDDC population appeared to be associated with the reduced level of expression on $CD86^+$ MDDCs (fig.IV.4b) as the P value was tending towards significance (P=0.07). Again, seminal plasma lowered the level of CD86 expression but did not reduce the proportion of $CD86^+$ MDDCs in MDDC population (R1) (fig.IV.4c). Decreases in expression of co-stimulatory molecules, in particular, CD86 were observed in 5 in 7 experiments and 2 showed no changes in expression of these molecules.

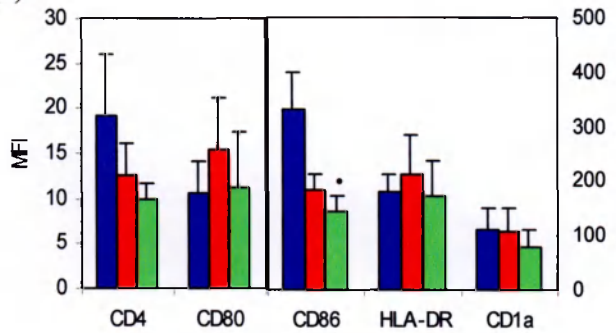
When MDDCs were exposed to TNF-α alone numbers of CD86 positive cells increased significantly (P=0.04) from 82.2(±3.6) to 92.6(±2.4): Δ% positive cells= 10.4%(±1.7) which is 13.1%(±2.6) (fig.IV.5c). TNF-α increased the levels of expression of CD86 on $CD86^+$ MDDCs but did not reach statistical significance as indicated by the P value (P=0.09; fig.IV.5b). All 5 experiments demonstrated increased levels of CD86 expression (MFI).

When MDDCs were pulsed overnight with both 200U/ml of TNF-α (10^6 cells/ml) and seminal plasma, expression of CD86 on these cells was increased

Fig.IV.4. Phenotypic analyses of seminal plasma (SPM) pulsed MDDCs

MDDCs were pulsed with two different concentrations (0.1%, 1%) of seminal plasma overnight. Cells were washed next day and stained for CD4, HLA-DR, CD1a, CD80 and CD86 as described in II.3.1. The graph (a) shows a MFI of expression of stated markers on a total MDDC population [R1 in (d)]. The graph (b) shows a mean fluorescence intensity (MFI) of expression of stated markers on positive MDDCs [M1 in (d)]with standard error. The graph (c) shows percentage of positive cells [M1 in (d)]. All the graphs show with standard errors for 8 (for CD4, HLA-DR and CD1a) and 6 (for CD80 and CD86) identical experiments. Tables next to graphs (a) and (b) show P values comparing MFI of each marker expressed on seminal plasma-pulsed MDDCs with that expressed on control MDDCs (ie. untreated MDDCs). The table next to the graph (c) shows P values comparing percentages of positive MDDCs expressing each marker in a MDDC population pulsed with seminal plasma with those in a control MDDC population. The graph (d) shows a dot plot of MDDC population and live cells are gated and designated as R1. Graphs (e), (f), (g), (h), and (i) are representatives of flow cytometry profile of CD4, CD80, CD86, HLA-DR, and CD1a, respectively. Expression of each marker was analysed in cells within the live cell population (R1). Positive markers (M1) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker.

(a)

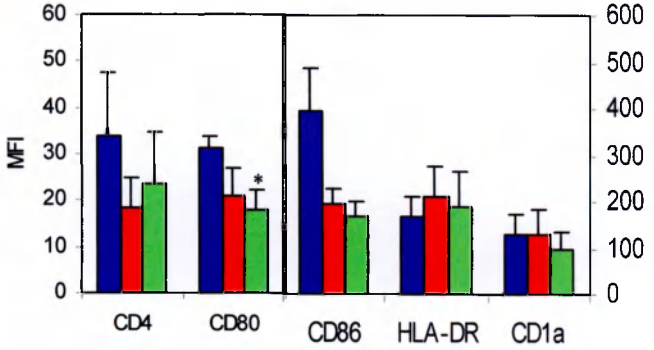


Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.4	0.6	0.1	**0.04***	0.09	0.0*	0.6	0.8	0.9	0.5
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.7		0.6		0.6		0.8		0.6	

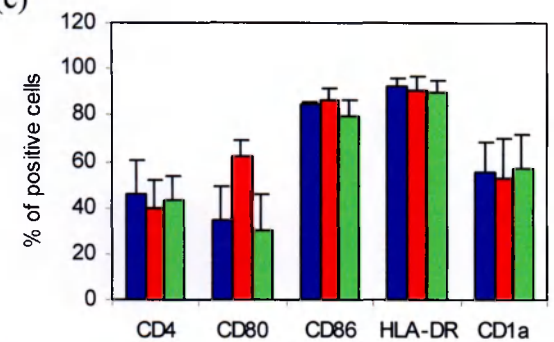
Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.5	0.2	0.4	0.9	0.09	**0.04***	0.6	0.9	0.9	0.5
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.5		0.6		0.4		0.7		0.6	

0.1%=0.1% SPM; 1%=1% SPM

(b)



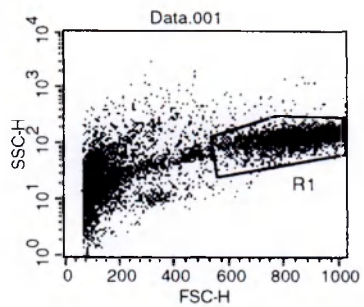
(c)



Control
0.1% SPM
1% SPM

Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.7	0.8	0.1	0.8	0.6	0.5	0.8	0.6	0.9	0.9
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.8		0.1		0.5		0.9		0.8	

(d)



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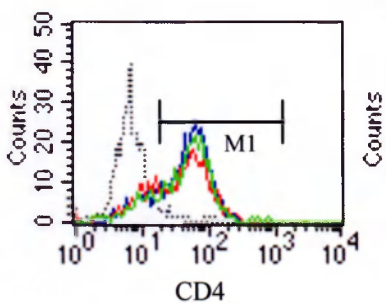

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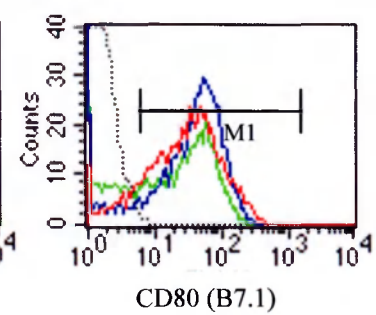

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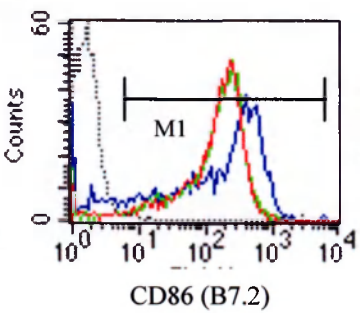

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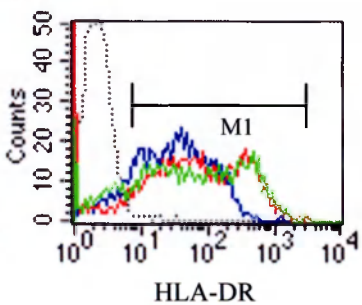


(i)

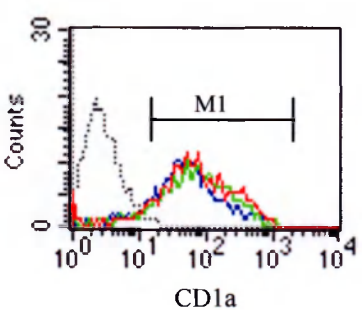
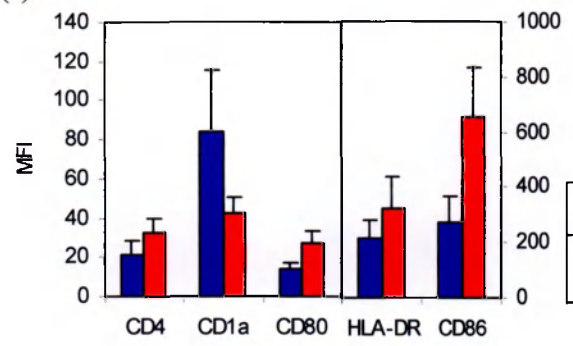


Fig.IV.5. Phenotypic analyses of TNF-α -exposed MDDCs

MDDCs were pulsed with 200U/ml of TNF-α overnight. Cells were washed next day and stained for CD4, HLA-DR, CD1a, CD80 and CD86 as described in II.3.1. The graph (a) shows a MFI of expression of stated markers on a total MDDC population [R1 in (d)]. The graph (b) shows a mean fluorescence intensity (MFI) of expression of stated markers on positive MDDCs [M1 in (d)]with standard error. The graph (c) shows percentage of positive cells [M1 in (d)]. All the graphs show with standard errors for 5 experiments. Tables next to graphs (a) and (b) show P values comparing MFI of each marker expressed on TNF-α -pulsed MDDCs with that expressed on control MDDCs (ie. untreated MDDCs). The table next to the graph (a) shows P values comparing percentages of positive MDDCs expressing each marker in a MDDC population exposed to TNF-α with those in a control MDDC population. The graph (d) shows a dot plot of MDDC population and live cells are gated and designated as R1. Graphs (e), (f), (g), (h), and (i) are representatives of flow cytometry profile of CD4, CD1a, CD80, HLA-DR, and CD86, respectively. Expression of each marker was analysed in cells within the live cell population (R1). Positive markers (M1) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker.

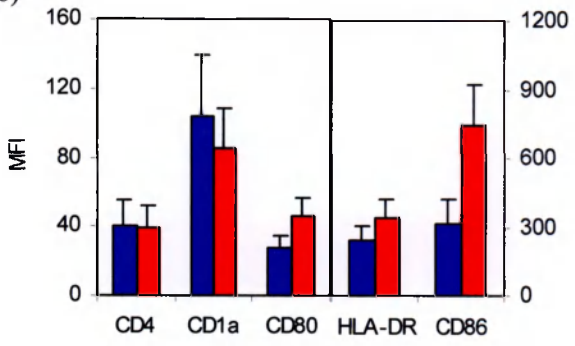
(a)

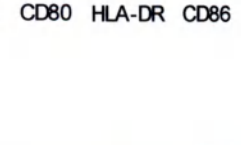


Marker	CD4	CD1a	CD80	HLA-DR	CD86
P values (vs. No TNF-α)	0.9	0.7	0.2	0.3	0.09

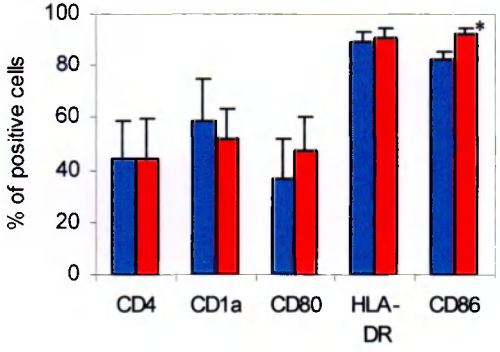
Marker	CD4	CD1a	CD80	HLA-DR	CD86
P values (vs. No TNF-α)	0.3	0.2	0.1	0.5	0.1

(b)



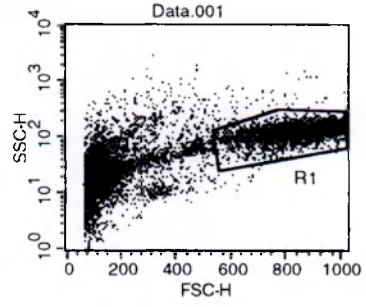


(c)

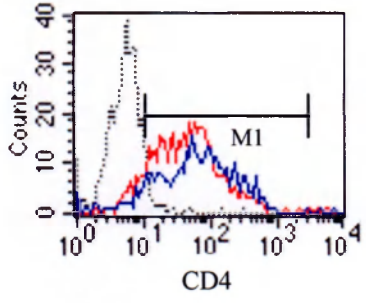


Marker	CD4	CD1a	CD80	HLA-DR	CD86
P values (vs. No TNF-α)	0.1	0.7	0.6	0.7	**0.04***

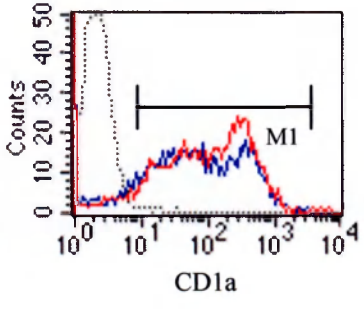
(d)



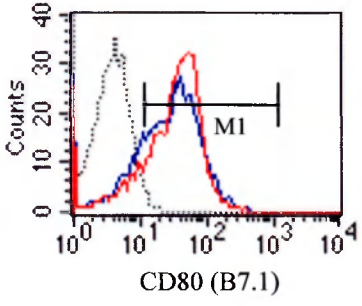
(e)



(f)



(g)



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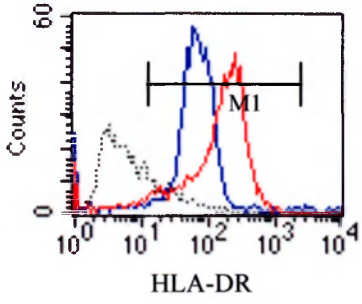


(i)

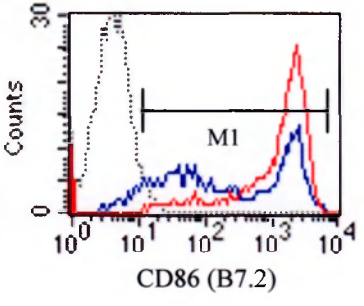
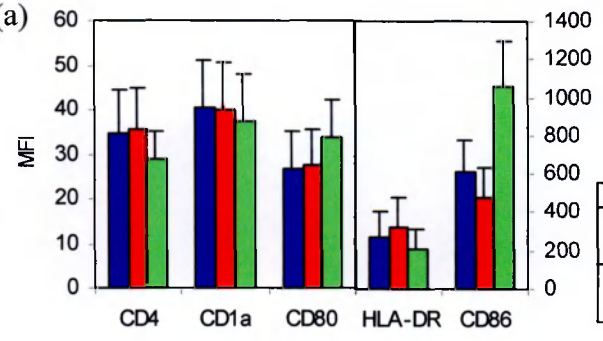


Fig.IV.6. Phenotypic analyses of MDDC exposed to both TNF-α and seminal plasma

MDDCs were pulsed with two different concentrations (0.1%, 1%) of seminal plasma together with 200U/ml of TNF-α overnight. Cells were washed next day and stained for CD4, HLA-DR, CD1a, CD80 and CD86 as described in II.3.1. The graph (a) shows a MFI of expression of stated markers on a total MDDC population [R1 in (d)]. The graph (b) shows a mean fluorescence intensity (MFI) of expression of stated markers on positive MDDCs [M1 in (d)]with standard error. The graph (c) shows percentage of positive cells [M1 in (d)]. All the graphs show with standard errors of 5 experiments. Tables next to graphs (a) and (b) show P values comparing MFI of each marker expressed on MDDCs exposed to both TNF-α and seminal plasma with that expressed on control MDDCs (ie. untreated MDDCs). The table next to the graph (c) shows P values comparing percentages of positive MDDCs expressing each marker in a MDDC population exposed to both TNF-α and seminal plasma with those in a control MDDC population. The graph (d) shows a dot plot of MDDC population and live cells are gated and designated as R1. Graphs (e), (f), (g), (h), and (i) are representatives of flow cytometry profile of CD4, CD1a, CD80, HLA-DR and CD86, respectively. Expression of each marker was analysed in cells within the live cell population (R1). Positive markers (M1) were placed on profiles so that less than 2% of cells stained with isotype control antibody fell within this marker.

(a)

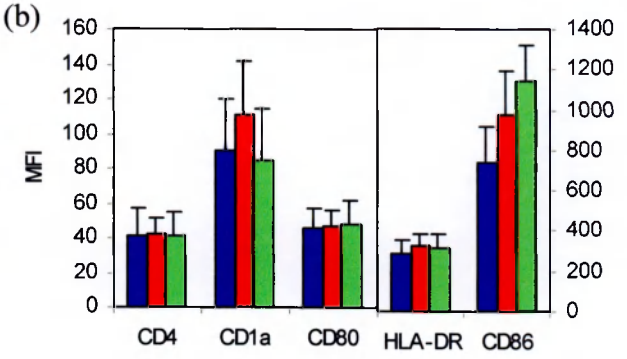


Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.9	0.9	0.9	0.8	0.4	0.2	0.7	0.9	0.6	0.9
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.9		0.9		0.6		0.8		0.6	

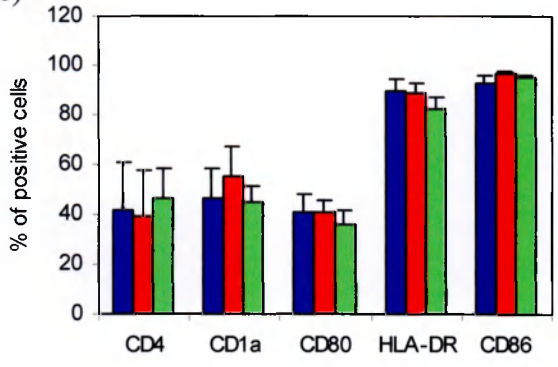
Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.9	0.6	0.8	0.6	0.6	0.2	0.6	0.7	0.9	0.8
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.6		0.6		0.3		0.6		0.9	

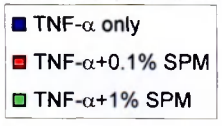
0.1%=0.1% SPM; 1%=1% SPM

(b)



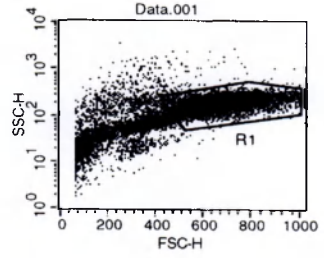
(c)



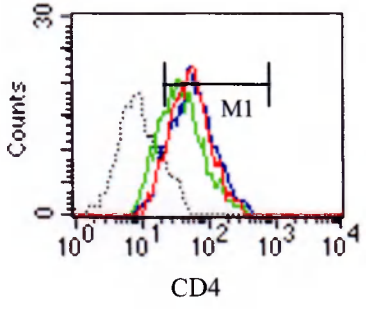


Marker	CD4		CD80		CD86		HLA-DR		CD1a	
P values (vs. control)	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%	1%
	0.8	0.2	0.9	0.6	0.3	0.4	0.9	0.3	0.6	0.9
P values (vs. 0.1% SPM)	1%		1%		1%		1%		1%	
	0.8		0.5		0.2		0.3		0.5	

(d)



(e)



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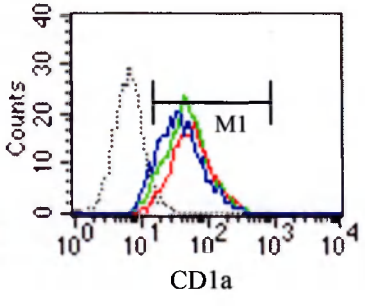

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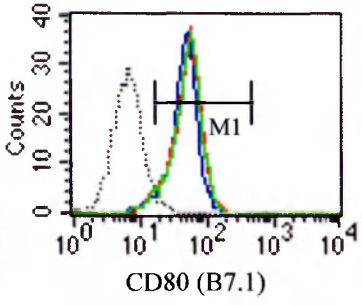

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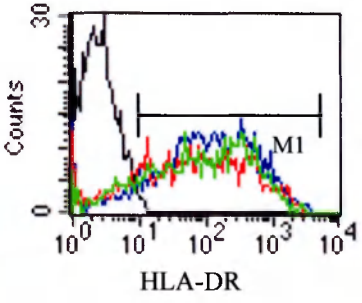
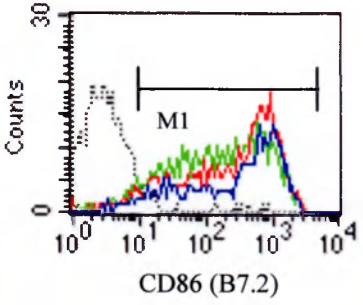


(i)



but not significantly (P=0.2; fig.IV.6a; P=0.2; fig.IV.6b) whereas the number of $CD86^+$ MDDCs remained unchanged, when compared with MDDCs pulsed with TNF-α only (fig.IV.6c). A decrease in the expression of co-stimulatory molecules induced by seminal plasma alone (fig.IV.4) was not seen when MDDCs were exposed to both TNF-α and seminal plasma together. 3 in 5 experiments demonstrated increased levels of CD86 by 1% seminal plasma. Taken together the data suggests that both TNF-α and seminal plasma alter the expression of co-stimulatory molecules on MDDCs and also TNF-α abrogates the effects of seminal plasma on MDDC phenotype. TNF-α alone increased (fig.IV.5) whilst seminal plasma alone decreased (fig.IV.4) co-stimulatory molecule expression. However, presence of seminal plasma did not induce any significant differences in the expression of co-stimulatory molecules on TNF-α stimulated MDDCs (fig.IV.6).

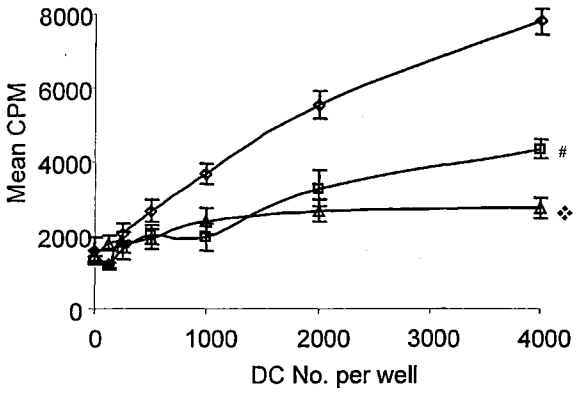
IV.2.3. Allostimulatory ability of DC treated with seminal plasma and/or TNF-α

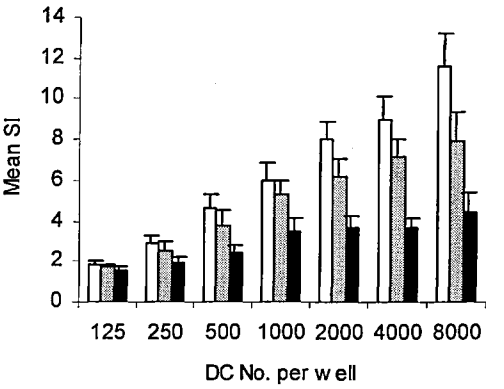
To assess APC function, seminal plasma-pulsed MDDCs were assessed for their ability to stimulate proliferation of allogeneic T cells. Fig.IV.7c describes a mean stimulation indices (SI) of 11 experiments and demonstrates that seminal plasma significantly suppressed the allostimulatory potentials of MDDCs at cell numbers of above 500. P values comparing the allostimulatory capacity of MDDCs which were pulsed with 1% seminal plasma, with that of control MDDCs, were less than 0.05 or tending towards significance (cell number of 8000) except when allogeneic T cells were stimulated by a small

Fig. IV.7. Allogeneic MLR with MDDCs pulsed with two different concentrations of seminal plasma

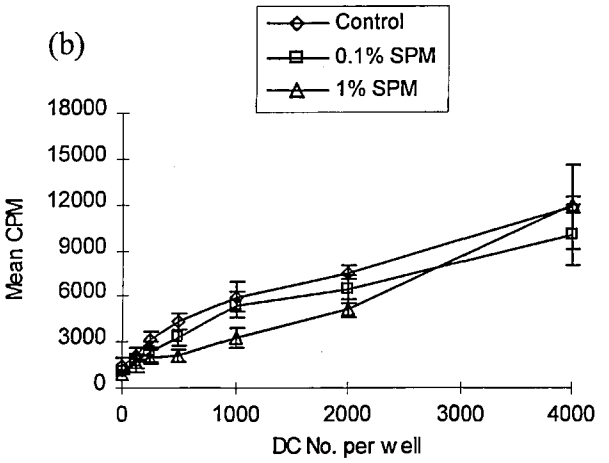
MDDCs were pulsed with 0.1% or 1% seminal plasma (SPM) overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs (a) show a representative of 7 experiments (left graph) and mean SI of these 7 experiments with SE (right graph) demonstrating suppression of allostimulatory potentials of MDDCs by seminal plasma. P values show that both 0.1% seminal plasma- and 1% seminal plasma-pulsed MDDCs demonstrated significantly reduced allostimulatory capacity. [P=0.003 (#): comparing allostimulatory capacity of 0.1% seminal plasma-pulsed MDDCs with that of control MDDCs; P=0.001 (❖): comparing allostimulatory capacity of 1% seminal plasma-pulsed MDDCs with that of control MDDCs]. Graphs (b) show a representative of 4 experiments (left graph) and mean SI of 4 experiments with SE (right graph) demonstrating no effect on allostimulatory potentials of MDDCs by seminal plasma. Graph (c) shows mean SI with SE of all 11 experiments. Each experiment was set up in triplicate. The table below graph (c) shows P values comparing ability of 0.1% or 1% seminal plasma-pulsed MDDCs to stimulate proliferation of allogeneic T cells with that of control, and P values comparing ability of 1% SPM-pulsed MDDCs to stimulate proliferation of allogeneic T cells with that of 0.1% SPM-pulsed MDDCs. P values both for the different number of MDDCs (125-8000) and for the trend curves (*italic*) are shown.

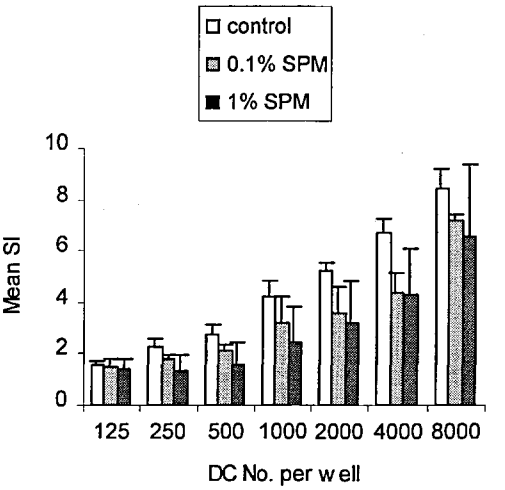
(a)



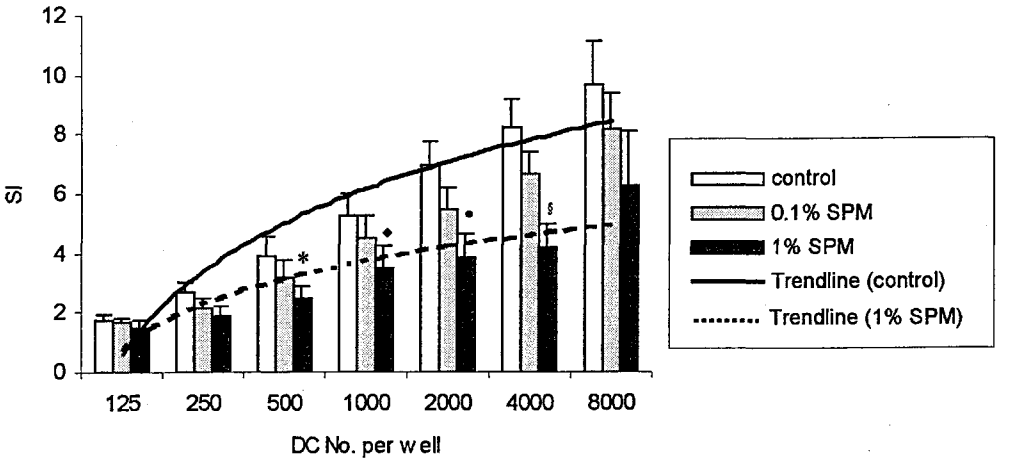


(b)





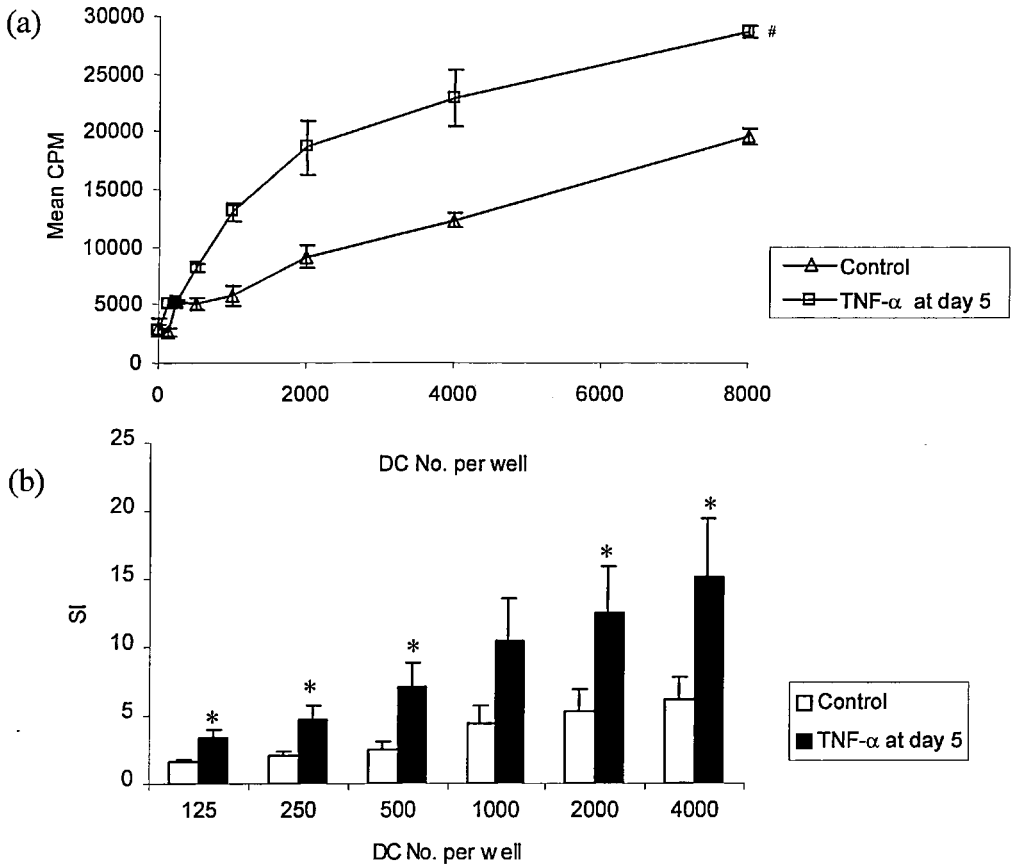
(c)



DC No. per well		125	250	500	1000	2000	4000	8000	*General trend*
P values (vs. control)	0.1% SPM	0.9	0.2	0.3	0.4	0.2	0.1	0.2	*0.1*
	1% SPM	0.4	0.1	**0.05***	**0.05**	**0.006**	**0.002**	0.09	***0.008***
P values (vs. 0.1% SPM)	1% SPM	0.4	0.7	0.4	0.3	0.1	0.07	0.4	*0.07*

number of DCs (cell number of less than 500). P values comparing the general trend in the allostimulatory capacity of MDDCs also showed that 1% seminal plasma-pulsed MDDCs have significantly reduced allostimulatory potentials (P=0.008). Allostimulatory potentials of MDDCs pulsed with 1% seminal plasma also decreased compared with 0.1% seminal plasma-pulsed MDDCs but not significantly (P=0.07). 7 experiments (fig.IV.7a; left graph: a representative of 7 experiments; right graph: mean SI of 7 experiments with SE) demonstrated suppression induced by seminal plasma (P=0.008 untreated vs. 0.1% SPM treated MDDCs; P=0.000, untreated vs. 1% SPM treated MDDCs) and 4 experiments (fig.IV.7b; left graph: a representative of 4 experiments; right graph: mean SI of 4 experiments with SE) demonstrated no effect on allostimulation capacity of MDDCs by seminal plasma (P>0.8, untreated vs. 0.1% or 1% seminal plasma treated MDDCs).

Significantly enhanced allostimulatory capacity of MDDCs treated with TNF-α alone compared with untreated MDDC was observed with greater than 95% confidence except when 1000 cells were used to stimulate allogeneic T cells (fig.IV.8b). Such a significant increase was observed in all 8 identical experiments and a single representative was presented (fig.IV.8a). The P value evaluated from the general trend curves (P=0.04) also showed a significant increase in allostimulatory potential of TNF-α treated MDDCs. Increased allostimulatory capacity of TNF-α treated MDDCs may result from the increased number of MDDCs expressing CD86 (fig.IV.5). Seminal plasma did not have any effect on the allostimulatory capacity of TNF-α stimulated MDDCs and this phenomenon was observed in all 6 experiments (fig.IV.9).



DC No. per well	125	250	500	1000	2000	4000
vs control	**0.03**	**0.05**	**0.05**	0.08	**0.05**	**0.05**

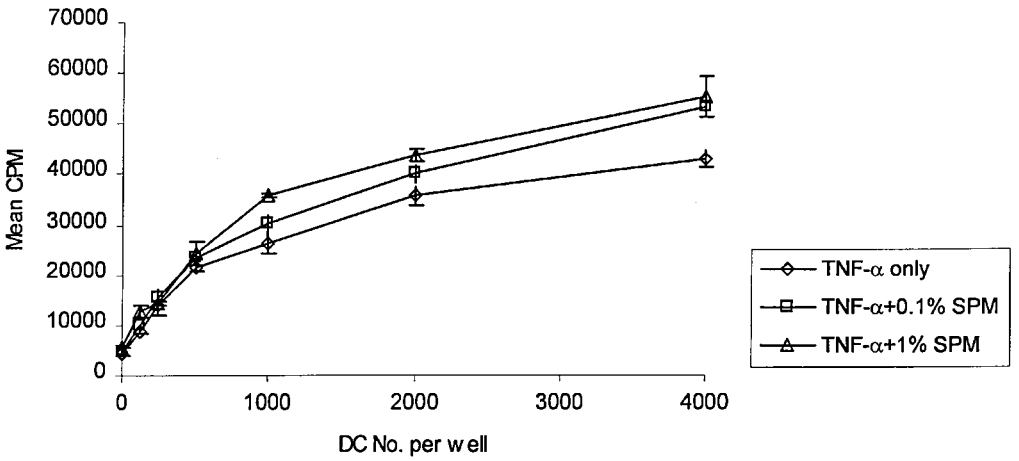
Fig. IV.8. Allogeneic MLR with MDDCs pulsed with TNF-α at day 5

MDDCs were pulsed with 200U/ml of TNF-α overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. The graph (a) is a representative of 8 experiments and shows mean counts per minute (CPM) with standard errors. The graph (b) shows mean stimulation indices (SI) with standard errors for 8 experiments. Each experiment was set up in triplicate. The table below the graph shows P values comparing SI of TNF-α-pulsed MDDCs with SI of control MDDCs (ie. not exposed to TNF-α). P(#)=0.009

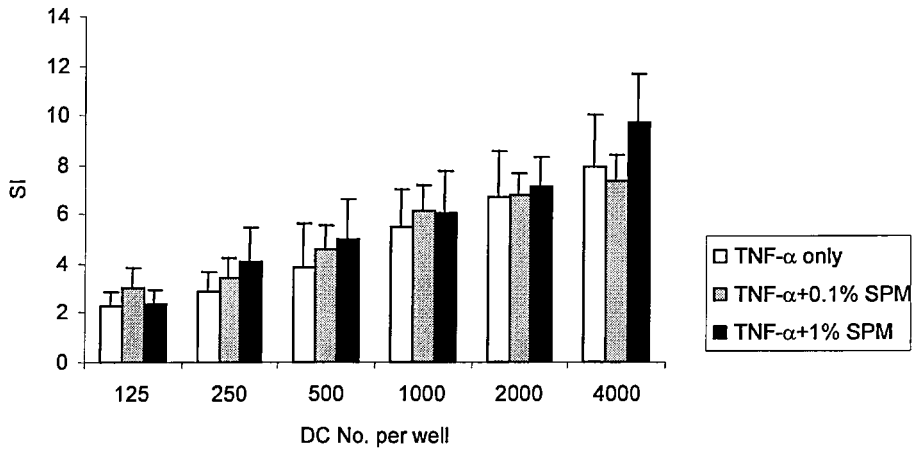
Fig. IV.9. Allogeneic MLR with MDDCs pulsed with TNF-α and two different concentrations of seminal plasma at day 5

MDDCs were pulsed with 200U/ml of TNF-α together with 0.1% or 1% seminal plasma (SPM) overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. The graph (a) shows a representative for 6 experiments and show mean counts per minute (CPM) with standard errors. Each experiment was set up in triplicate. The graph (b) shows mean stimulation indices (SI) with standard errors of 5 experiments. The table below graph (b) shows P values comparing ability of TNF-α/0.1% SPM-pulsed DCs or TNF-α/1% SPM-pulsed MDDCs to stimulate proliferation of allogeneic T cells with that of MDDCs pulsed with TNF-α only. P values for allostimulatory capacity of MDDCs pulsed with both TNF-α and seminal plasma at different MDDC numbers (125-4000) and for that comparing the general trends of each curve (*italic*) are shown.

(a)



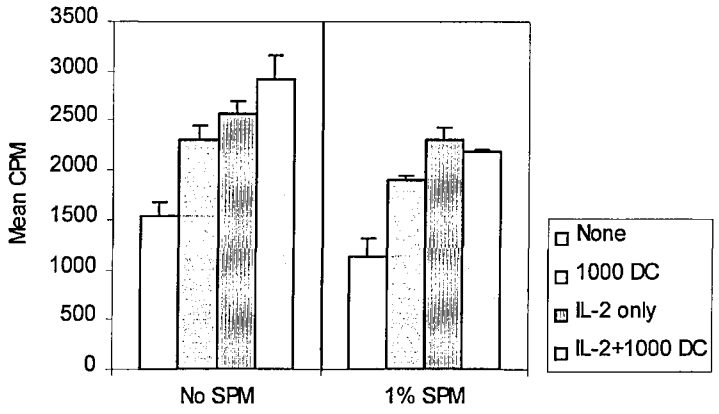
(b)



DC No. per well		125	250	500	1000	2000	4000	*General trend*
P values (vs. TNF-α only)	TNF-α+0.1% SPM	0.5	0.6	0.7	0.8	0.9	0.9	*0.7*
	TNF-α+1% SPM	0.8	0.4	0.4	0.7	0.6	0.3	*0.2*
P values (vs. TNF-α+0.1% SPM)	TNF-α+1% SPM	0.6	0.6	0.6	0.9	0.6	0.2	*0.3*

When MDDCs were exposed to both TNF-α and seminal plasma the suppressive effect of seminal plasma on the allostimulatory ability of these cells was no longer observed. These data suggest that TNF-α could block or overcome the suppressive effect of seminal plasma. This phenomenon is associated with phenotypic analyses which demonstrated the increased number of $CD86^{+}$ MDDCs upon TNF-α stimulation (fig.IV.5) and the presence of seminal plasma did not induce any alteration in the phenotype of TNF-α stimulated MDDCs (fig.IV.6). Pro-inflammatory cytokines, like TNF-α, play a role in activation and maturation of DCs. Mature DCs which are seen in the lymph node have higher ability to stimulate T cells compared with immature DCs in the mucosa.

Whether seminal plasma-induced suppression of the allostimulatory ability of MDDCs may be due to induction of anergic T cells was investigated. Fig.IV.10 demonstrates that IL-2, fresh MDDCs which have never been exposed to seminal plasma, or both together restored the proliferation of allogeneic T cells previously exposed to seminal plasma-treated MDDCs, indicating that the suppression of proliferation of allogeneic T cells induced by the seminal plasma-pulsed MDDCs is unlikely to be due to the induction of anergic T cells.



DC treatment	No SPM	1% SPM
1000 DCs only	0.03	0.03
IL-2 only	0.01*	0.008*
IL-2+1000 DCs	0.02	0.02

Fig.IV.10. Recovery of cell proliferation

The MDDCs were pulsed with 1% seminal plasma overnight. Seminal plasma-pulsed MSDCs (right column) and MDDCs which were not exposed to seminal plasma (left column) were washed and an allogeneic MLR was set up next day and cultured for 3 days. Cells were washed, counted and secondary MLR was set up using 10^5 cell/well from the first MLR as responder cells. Additives to the secondary MLR were i) 9 days-old MDDCs (1000 cells/well) from the same donor as that used in the first MLR (▨), ii) 20 units of IL-2 (▥), or iii) both MDDCs and IL-2 together (⬚). As a control, nothing was added (□). Cells were further cultivated for 3 days and washed, pulsed with 0.5μCi of [3H]-thymidine per well next day. The graph shows mean counts per minutes with standard errors of 3 experiments. Each experiment was set up in triplicate. The table below the graph shows P values comparing allogeneic T cell proliferation with and without additives.

IV.2.4. Identification of seminal components responsible for suppression of allostimulatory ability of MDDC

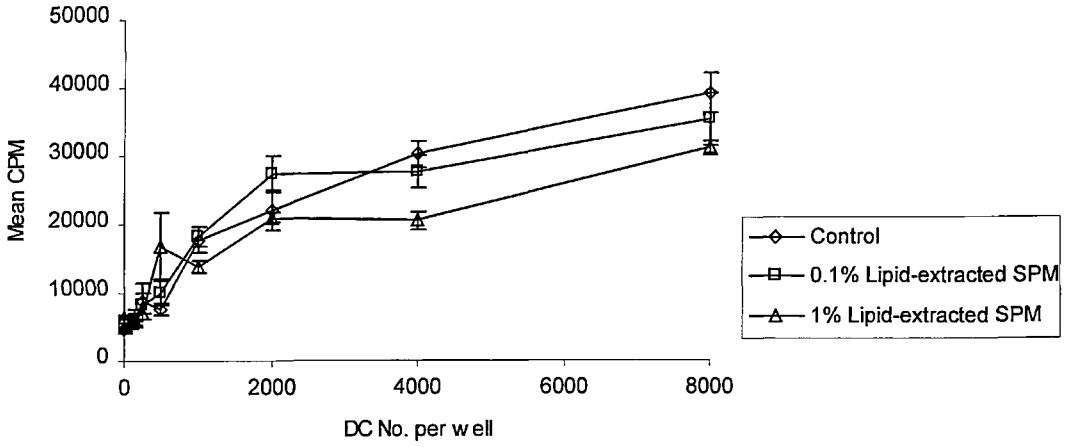
Lipids were extracted from seminal plasma using a reverse phase column as described in II.2.2. Allogeneic MLRs were set up using MDDCs which were pulsed overnight with lipid-extracted seminal plasma. By extracting lipids from seminal plasma, most of prostaglandins are eliminated from the seminal plasma (Kelly *et al*, 1997[a]). Such experiments allowed examination of the role of prostaglandins in the alteration of seminal plasma-pulsed MDDCs' function. Fig.IV.11 demonstrates that lipid extraction abrogated the suppressive effect of seminal plasma on MDDCs (previously seen in fig.IV.8). No significant differences between three groups of MDDCs (i.e. untreated, 0.1% lipid-extracted seminal plasma treated, and 1% lipid-extracted seminal plasma treated MDDCs) were detected ($P>0.05$). These findings suggest that lipids in seminal plasma could be responsible for suppression of allostimulatory ability.

To further identify whether PGE2, in particular, is responsible for suppressive effect of seminal plasma, MDDCs were pulsed with exogenous PGE2 instead of seminal plasma at day 5. It is known that prostaglandins which are present in human semen at higher concentrations than in peripheral blood (Kelly, 1997[a]), have immunomodulatory effects including ability to induce tolerogenic DCs which are also known as type-3 polarised effector DCs (Steinbrink *et al*, 1997) and to induce type-2 effector DCs which have capacity to stimulate T cells efficiently Kalinstki *et al*, 1997; Kalinski *et al*, 1998). The average

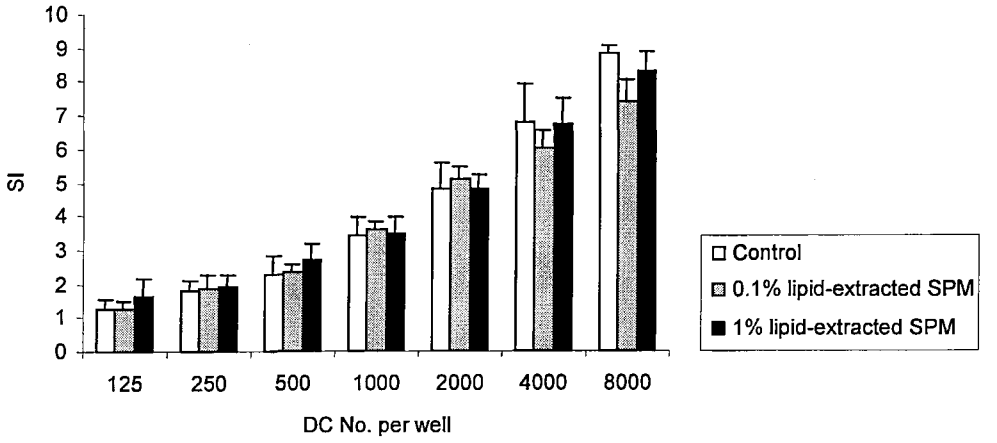
Fig.IV.11. Allogeneic MLR with MDDCs pulsed with two different concentrations of lipid-extracted seminal plasma

MDDCs were pulsed with 0.1% or 1% lipid-extracted seminal plasma (SPM) overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. The graph (a) shows a representative of 3 experiments and shows mean counts per minute (CPM) with standard errors. The graph (b) shows mean stimulation indices (SI) with standard error for 3 experiments. Each experiment was set up in triplicate. The table below graph (b) shows P values comparing SI of MDDCs pulsed with 0.1% or 1% lipid-extracted SPM with SI of control MDDCs (ie. untreated DCs).

(a)



(b)



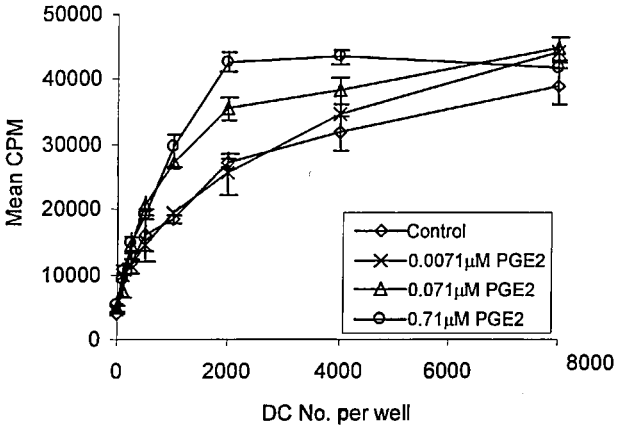
DC No. per well		125	250	500	1000	2000	4000	8000
vs control	0.1% lipid-extracted SPM	0.9	0.9	0.9	0.8	0.8	0.6	0.1
	1% lipid-extracted SPM	0.6	0.8	0.6	0.9	0.9	0.9	0.4
vs 0.1% lipid-extracted SPM	1% lipid-extracted SPM	0.6	0.9	0.6	0.8	0.6	0.5	0.6

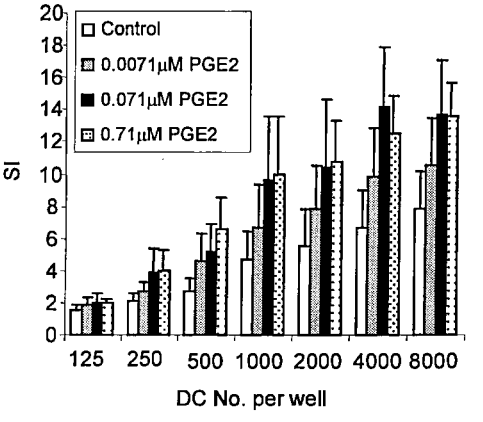
concentration of PGE2 in seminal plasma is about 25μg/ml (=71μM) and that of 19-OH PGE2 is about 130μg/ml (=0.353mM) (Kelly *et al*, 1997). MDDCs were exposed to graded doses of PGE2 [0.0071μM-0.71μM which is equivalent to 0.001%-1% seminal plasma (fig.IV.12)]. Exogenous PGE2 did not induce the same effect on MDDCs as seminal plasma. PGE2 enhanced the allostimulatory ability of MDDCs (fig.IV.12a). When TNF-α was added together with PGE2 increased proliferation of allogeneic T cells was observed at a low number of MDDCs, but overall such an increment was not significant as indicated by P values evaluated from trend lines (fig.IV.12b). Neither 19-OH PGE2 (0.0353μM-3.53μM which is equivalent to 0.001%-1% seminal plasma) alone, nor 19-OH PGE2 together with TNF-α have significant effects on allostimulatory ability of MDDCs (fig.IV.13). In one experiment an increased allostimulatory ability of MDDCs pulsed with 3.53μM of 19-OH PGE2 was demonstrated (P=0.004; fig.IV.13a, left hand graph). These data suggests that although lipids in seminal plasma may play a role in the suppressive effect on allostimulatory potential of MDDCs, we failed to show the direct involvement of either PGE2 or 19-OH PGE2. It is possible that the suppressive effect of seminal plasma may be a result of combined activities of prostaglandins and other immunosuppressive components present in seminal plasma and hence exogenous prostaglandins do not have the same effect as seminal plasma on the allostimulatory potentials of MDDCs.

Fig. IV.12. Allogeneic MLR with MDDCs pulsed with PGE2

MDDCs were pulsed with graded doses of PGE2 (0.0071μM-0.71μM) alone (a) or together with 200U/ml of TNF-α (b) overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Histograms on the left show mean stimulation indices (SI) with standard errors for 4 experiments. Graphs on the right are single representatives of 4 experiments and show mean counts per minute (CPM) with standard error. Each experiment was set up in triplicate. The table below IV.12a shows P values comparing SI of MDDCs pulsed with PGE2 to stimulate proliferation of allogeneic T cells with that of control MDDCs (ie. untreated MDDCs). The table below IV.12b shows P values comparing SI of MDDCs pulsed with PGE2 together with TNF-α with SI of MDDCs pulsed with TNF-α only.

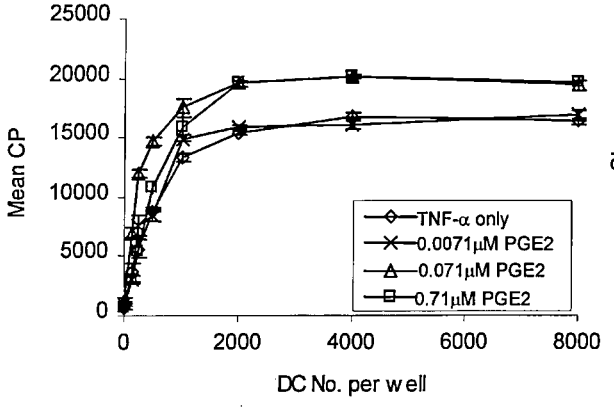
(a)

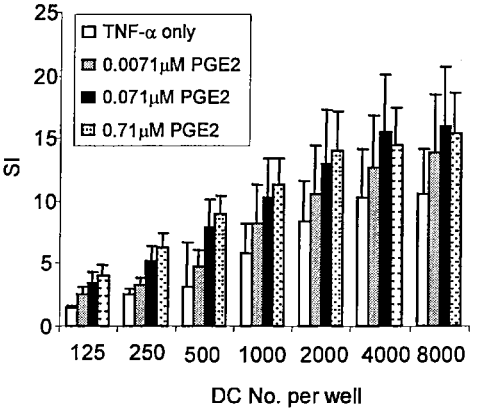




DC No. per well		125	250	500	1000	2000	4000	8000
vs control	0.0071μM PGE2	0.6	0.5	0.4	0.5	0.5	0.4	0.5
	0.071μM PGE2	0.5	0.3	0.3	0.3	0.3	0.3	0.2
	0.71μM PGE2	0.3	0.3	0.2	0.2	0.2	0.1	0.1
vs 0.0071μM PGE2	0.071μM PGE2	0.8	0.5	0.8	0.6	0.6	0.7	0.5
	0.71μM PGE2	0.8	0.4	0.5	0.5	0.5	0.5	0.4
vs 0.071μM PGE2	0.71μM PGE2	0.9	0.9	0.7	0.9	0.9	0.9	0.9

(b)



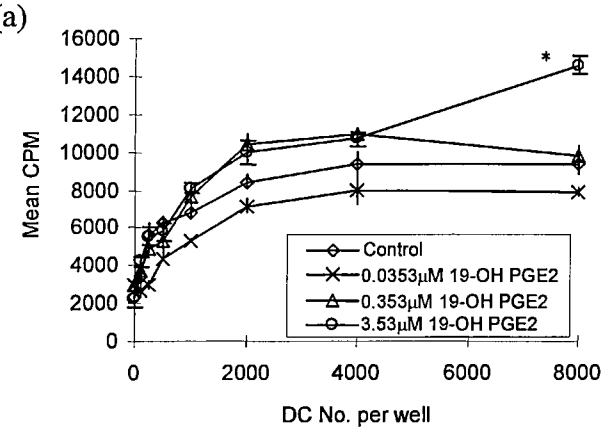


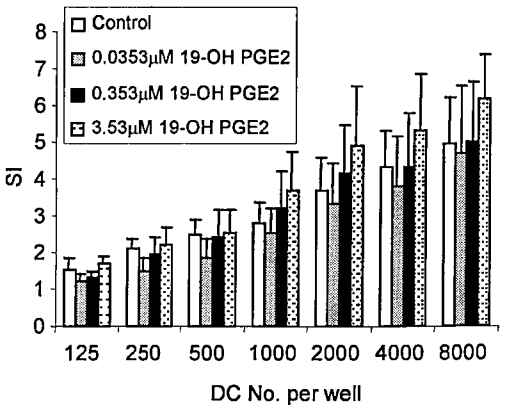
DC No. per well		125	250	500	1000	2000	4000	8000	*General trend*
vs TNF-α only	TNF-α+0.0071 μM PGE2	0.2	0.4	0.3	0.5	0.7	0.7	0.6	*0.7*
	TNF-α+0.071 μM PGE2	0.08	0.09	0.1	0.3	0.4	0.4	0.4	*0.6*
	TNF-α+0.71 μM PGE2	**0.05**	**0.04**	**0.01**	0.1	0.3	0.4	0.3	*0.7*
vs. TNF-α+ 0.0071 μM PGE2	TNF-α+0.071 μM PGE2	0.4	0.2	0.3	0.7	0.7	0.7	0.8	*0.9*
	TNF-α+0.71 μM PGE2	0.2	0.09	0.08	0.4	0.5	0.7	0.8	*0.9*
vs TNF-α+0.071 μM PGE2	TNF-α+0.71 μM PGE2	0.7	0.6	0.7	0.8	0.9	0.9	0.9	*0.8*

Fig. IV.13. Allogeneic MLR with MDDCs pulsed with 19-OH PGE2

MDDCs were pulsed with graded doses of 19-OH PGE2 (0.0353μM-3.53μM) alone (a) or together with 200U/ml of TNF-α (b) overnight. Cells were washed next day, an allogeneic MLR was set up and cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs on the right show mean stimulation index (SI) with standard errors for 4 experiments. Graphs on the left are single representatives of 4 experiments and show mean counts per minute (CPM) with standard errors. Each experiment was set up in triplicate. The tables below IV.13a show P values comparing SI of MDDCs pulsed with 19-OH PGE2 with SI of control MDDCs (ie. untreated MDDCs). The table below IV.13b shows P values comparing SI of MDDCs pulsed with 19-OH PGE2 together with TNF-α with SI of MDDCs pulsed with TNF-α only. P=0.04 (*)

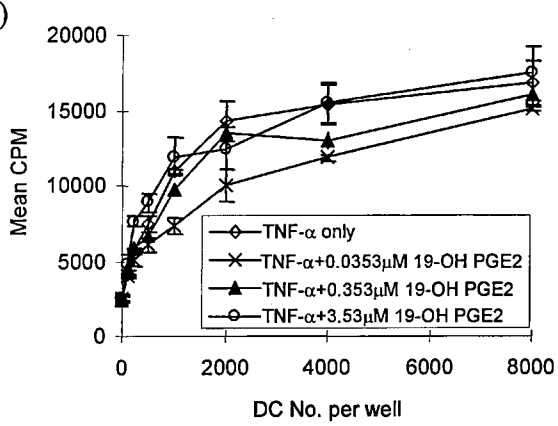
(a)

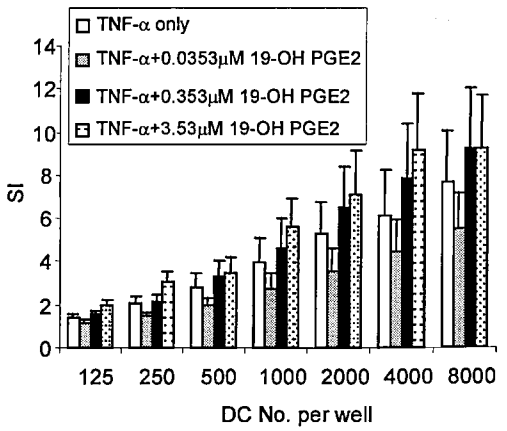




DC No. per well		125	250	500	1000	2000	4000	8000
vs control	0.0353μM 19-OH PGE2	0.4	0.2	0.4	0.8	0.8	0.8	1.0
	0.353μM 19-OH PGE2	0.6	0.8	0.9	0.7	0.8	1.0	1.0
	3.53μM 19-OH PGE2	0.7	0.8	0.9	0.5	0.5	0.6	0.5
vs 0.0353μM 19-OH PGE2	0.353μM 19-OH PGE2	0.6	0.4	0.6	0.6	0.6	0.8	1.0
	3.53μM 19-OH PGE2	0.2	0.2	0.4	0.4	0.4	0.5	0.6
vs 0.353μM 19-OH PGE2	3.53μM 19-OH PGE2	0.2	0.7	0.9	0.7	0.7	0.7	0.6

(b)





DC No. per well		125	250	500	1000	2000	4000	8000
vs TNF-α only	TNF-α+0.0353 19-OH PGE2	0.4	0.2	0.3	0.4	0.4	0.6	0.5
	TNF-α+0.353μM 19-OH PGE2	0.5	0.8	0.7	0.7	0.6	0.6	0.7
	TNF-α+3.53μM 19-OH PGE2	0.2	0.2	0.3	0.4	0.5	0.4	0.7
vs 0.0353μM 19-OH PGE2	TNF-α+0.353μM 19-OH PGE2	0.2	0.1	0.2	0.3	0.2	0.3	0.3
	TNF-α+3.53μM 19-OH PGE2	0.06	**0.03**	0.06	0.1	0.2	0.2	0.3
vs 0.353μM 19-OH PGE2	TNF-α+3.53μM 19-OH PGE2	0.3	0.2	0.6	0.6	0.8	0.7	1.0

IV.3. DISCUSSION

In this part of thesis, the effect of seminal plasma and/or TNF-α on the phenotype and functional ability to stimulate allogeneic T cells of the $CD14^+$ monocyte-derived DCs (MDDCs) was studied. It has been well established that DCs generated from monocytes using GM-CSF and IL-4 have the properties of immature DCs since they have a high capacity for endocytosis and low T cell stimulation function (Sallusto, *et al*, 1995; Sallusto and Lanzavecchia, 1994). To assess APC function, MDDCs were tested for their ability to stimulate proliferation of allogeneic T cells since DCs always carry allo-peptides.

TNF-α induced maturation of MDDCs as demonstrated by assessment of allostimulatory capacity of the cells (fig.IV.8). When TNF-α was present in the MDDC culture, the cells showed increased allostimulatory capacity compared with those which had not been treated with TNF-α. It has been shown that inflammatory cytokines and bacterial products can stimulate DC maturation and migration as has been demonstrated by systemic administration of TNF-α, IL-1β and IL-6 (Sallusto and Lanzavecchia, 1994; Sallusto *et al*, 1995; Jonuleit *et al*, 1997; Cumberbaten *et al*, 1990) or LPS (Cella, *et al*, 1997; Roake, *et al*, 1995) which induced depletion of DCs from non-lymphoid organs and migration into lymph nodes. DCs produce ceramides on stimulation by TNF-α, IL-1 and CD40L and ceramides can inhibit endocytosis which is a characteristic of immature DCs (Sallusto *et al*, 1996). IL-1 (Kobayash *et al*, 1989), IL-6 and TNF-α (Okamoto *et al*, 1989) activate NF-

κB. Mature DCs express high levels of the NF-κB family of transcriptional control proteins which regulate the expression of immune and inflammatory proteins. This, consequently, may induce further activation of DCs and may also induce migration of other immune cells to the site of inflammation. Phenotypic analyses showed that TNF-α significantly increased numbers MDDCs expressing one of co-stimulatory molecules, CD86/B7.2 (fig.IV.6b) which are involved in presentation of antigen in the context of MHC class II to CD4 helper T cells. However, TNF-α did not increase expression of HLA-DR or decrease CD1a which are thought to occur during maturation of DCs.

Seminal plasma reduced the expression of co-stimulatory molecules on MDDCs (fig.IV.4). These data were supported by data obtained from functional ability of MDDCs to stimulate allogeneic T cells (fig.IV.7). Lower concentration of seminal plasma used in this study (0.1%) did not have any effect on neither the phenotype nor the allostimulatory function of MDDCs. However, the suppression did not appear to be due to induction of anergic T cells by seminal plasma since addition of IL-2, fresh MDDCs (i.e. not previously exposed to seminal plasma) or both together restored the proliferation of allogeneic T cells (fig.IV.10). Investigation of the cytokine production by seminal plasma-pulsed MDDCs is required to further characterise the cells. Increased IL-10 and decreased IL-12 production may induce anergy (Groux *et al*, 1996; Van Parijs *et al*, 1997). When MDDCs were exposed to TNF-α, the suppressed allostimulatory ability of the cells which was induced by seminal plasma was no longer observed. There was not any significant difference in neither phenotypes nor allostimulatory potentials of

MDDCs. This data suggests that TNF-α counterbalances the effect of seminal plasma or *vice versa*. This phenomenon may be what is expected *in vivo*. The immature mucosal DCs do not recognise the seminal cells as foreign and semen does not induce immune response against itself. Pro-inflammatory cytokines like TNF-α may be present at a high level in semen of patients with genitourinary (GU) infections (Ramsey *et al*, 1995) and may act as a 'danger' signal (Matzinger, 1994). The results presented in this study suggest that under such circumstances the masked function of mucosal DCs as APCs which is induced by semen is blocked and hence, are ready to present the pathogens in the semen but whether the mucosal DCs recognise seminal components as foreign antigen in such scenarios requires to be investigated.

It has been shown that PGE2 and 19-OH PGE2 which are the main prostaglandin constituents of human seminal plasma induce Th2 type of responses (Kelly *et al*, 1997[b]; Kalinski, *et al*, 1997; Groux *et al*, 1996; Kalinski, *et al*, 1998) and may induce anergy (Groux *et al*, 1996; Mannie *et al*, 1995; Phipps and Scott, 1983; Goldings *et al*, 1986). The effect of seminal plasma on allostimulatory capacity of MDDCs was abrogated when the cells were treated with lipid-extracted seminal plasma (fig.IV.10) suggesting that lipids in semen may play a role in altering the function of the cells. To further identify whether PGE2 and/or 19-OH PGE2 are the key component responsible for the suppressive effect of seminal plasma, experiments using commercially available exogenous PGE2 and 19-OH PGE2 were performed. The results showed that exogenous prostaglandins do not have the same effect as seminal plasma (fig.IV.12 & 13). Our data was supported by studies by

Riesser and her colleagues who have shown that PGE2 alone activated DCs inducing IL-12 production in DCs (Rieser, *et al*, 1997). PGE2 affects development of immature DCs. The presence of PGE2 in immature DC culture results in induction of type 2-polarised effector DCs which produce reduced amounts of IL-12 and hence induces a bias towards Th2 type cytokine production in naïve T cells (Kalinski *et al*, 1997; Kalinski *et al*, 1998). These type 2-polarised effector DCs induced by PGE2 have enhanced stimulatory potential (Kalinski *et al*, 1998). The data from our study demonstrated an increased allostimulatory potential of MDDCs by PGE2 or 19-OH PGE2 although it was not statistically significant. Taken together it suggests that there are other factors in seminal plasma responsible for the functional changes of DCs, such as prostasome, polyamine, TGF-β and IL-10 although IL-10 present in semen at a very low concentration (Kelly, 1995; Alexander and Ansderson, 1985). Although prostaglandin by itself did not induce the suppressive effect *in vitro* it cannot be ruled out the possibility that the suppressive effect of seminal plasma may be the result of combined activity of prostaglandin and other components present in semen.

In conclusion the data from this chapter has shown that semen has immunomodulatory properties on MDDCs. Seminal plasma alone suppressed antigen presentation of MDDCs and this effect was abrogated when pro-inflammatory cytokines such as TNF-α were present. This has important implications. Seminal plasma has a role in protecting sperm from recognition in the female reproductive tract by possibly switching to Th2-type responses which may induce anergy or tolerance (Kelly *et al*, 1997[a]). However, when

high levels of pro-inflammatory cytokines are present in semen, as may be found in patients with GU infections (Ramsey *et al*, 1995), the cytokines and pathogens in semen act as 'danger' signals (Matzinger, 1994) and may induce activation of LCs in the mucosa resulting in enhanced ability to present antigens and to stimulate T cells in the lymph node. More importantly, under such circumstances seminal plasma may not play a protective role any longer. Possible mechanisms for the suppressive effect of seminal plasma and for the way in which TNF-α may overcome the suppressive effect of seminal plasma are summarised in fig.IV.14.

Future work

1. Detection of IL-10 in seminal plasma

It has been shown that IL-10 has an immunomodulatory effect on immature DCs. IL-10 inhibits both IL-12 production in immature DCs (De Smedt *et al*, 1997; Kalinski *et al*, 1998) and the stimulatory ability of DCs, possibly by down-regulating expression of co-stimulation molecules (Chang *et al*, 1995; Willems *et al*, 1994; Ding *et al*, 1993) and thus inducing the development of a tolerogenic type of DC (Steinbrink *et al*, 1997). Since seminal plasma-pulsed MDDCs had a suppressed capacity for allostimulation, the presence of IL-10 in semen needs to be studied.

2. Measurement of level of prostaglandins in seminal plasma

Since prostaglandins (PGE2 and 19-OH PGE2) have been shown to have an immuno-suppressive effect (Kraan *et al*, 1995; Kelly *et al*, 1997[b]) the amount of 19-OH PGE2 in individual semen samples needs to be measured. Also

individual lipids separated from seminal plasma using a reverse phase column could be eluted and used to treat DCs in the place of seminal plasma.

3. Cytokine production by DCs and T cells

Whether seminal plasma and/or TNF-α induces changes in cytokine production by DCs needs to be studied. Several studies have shown that DCs are important inducers of Th1 and Th2 cytokines in naïve T cells and such discrimination appears to be due to the level of IL-12 production rather than the ability to produce IL-12 (Macatonia *et al*, 1993; Hilkens *et al*, 1997; Ronchese *et al*, 1994; Stumbles *et al*, 1998; Snijders *et al*, 1998). This is of importance since the different types of Th cells induce different immune responses. Th1-type cytokines promote cellular immunity and Th2-type cytokines support humoral immunity. The type of cytokines produced by T cells exposed to seminal plasma-pulsed DCs could be evaluated. Intracellular cytokine staining appeared to be not sensitive enough to detect IL-10 or IL-12 in DCs (data not shown). Other methods such as ELISA or bioassay could be used to measure cytokine production by DCs and by allogeneic T cells after stimulation with DCs.

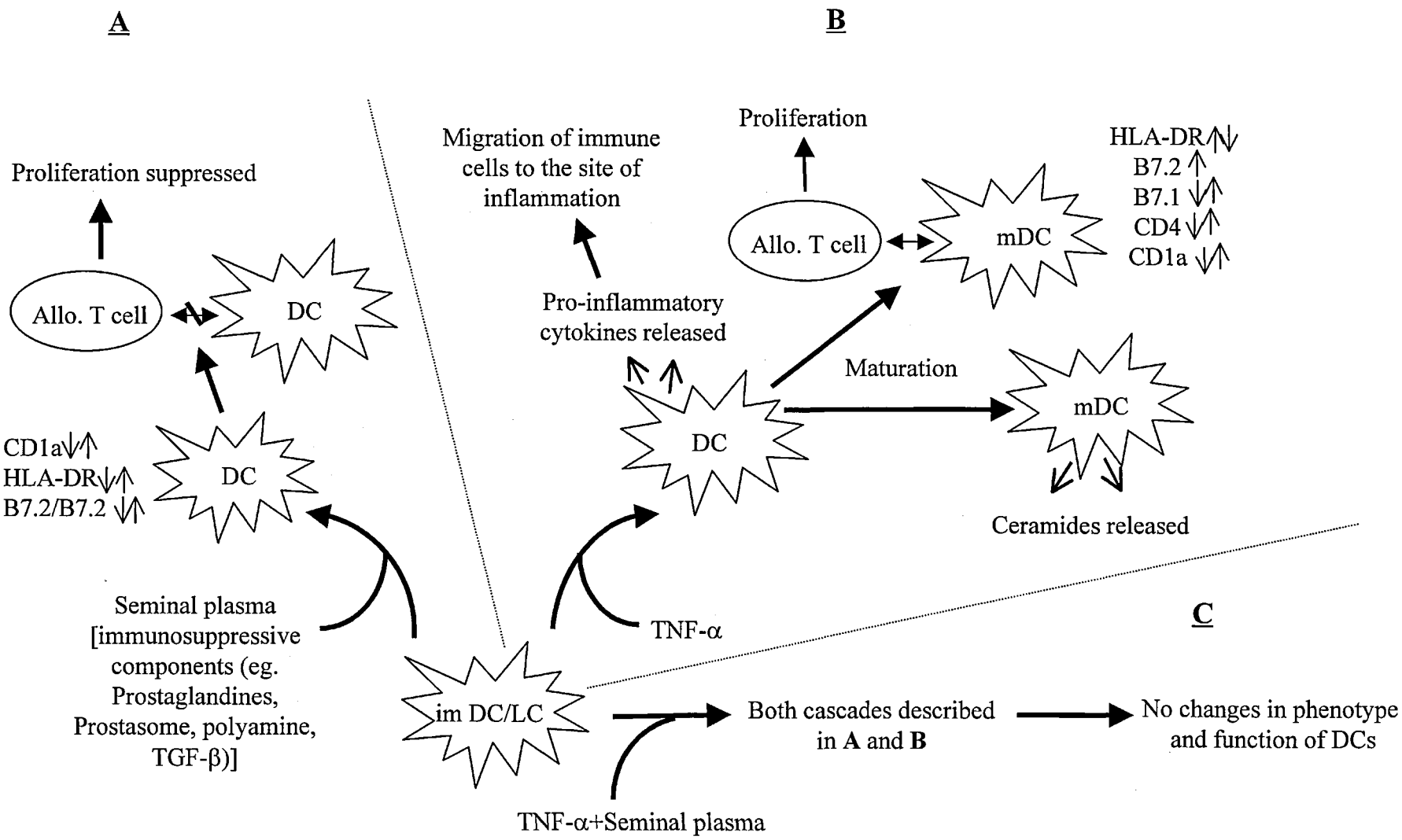
4. Antigen-uptake capacity of DCs

To complete the functional studies on seminal plasma- and/or TNF-α-pulsed DCs, the antigen-uptake capacity of these cells needs to be assessed using fluorescien-conjugated dextran or nitroblue tetrazolium/zymosan granules, as has been previously described (Robinson *et al*, 1999). Antigen-uptake by DCs is as important as stimulatory capacity of DCs in terms of DC function.

Fig.IV.14. Possible mechanisms for altered allostimulatory capacity of DCs which are exposed to seminal plasma and DCs which are exposed to TNF-α and for TNF-α overcoming the suppressive effect of seminal plasma

Seminal plasma alone reduced expression of co-stimulatory molecules on DCs and suppressed allostimulatory ability of DCs significantly (**A**). Semen contains immunosuppressive components [eg. Prostaglandins, prostasomes, polyamine and TGF-β (Alexander and Anderson, 1987; Kelly, 1995)]. TNF-α alone had the opposite effect on phenotyoe and function of DCs (**B**). TNF-α induces maturation of DCs since TNF-α induces ceramide release in DCs resulting in inhibition of endocytosis. TNF-α also induces secretion of pro-inflammatory cytokines by DCs as a result of activation of NF-κB (Kobayash *et al*, 1989; Okamoto *et al*, 1989). Secretion of pro-inflammatory cytokines may induce migration of other immune cells to the site of inflammation *in vivo* and may also induce further maturation of DCs themselves. TNF-α may overcome the suppressive effect of seminal plasma by inducing maturation of DCs as described above, however seminal plasma also counteracts with the effect of TNF-α. Thus, this counterbalancing may have resulted in unchanged phenotype and function of DCs (**C**).

(imDC: immature DC; mDC: mature DC; ➡: induction; ⟷: interaction; ➔: secretion; ↑: increase; ↓ : decrease; ⇅: no change)


A
B
Proliferation suppressed
Allo. T cell
DC
CD1a
HLA-DR
B7.2/B7.2
DC
Seminal plasma
[immunosuppressive
components (eg.
Prostaglandines,
Prostasome, polyamine,
TGF-β)]
Migration of immune
cells to the site of
inflammation
Pro-inflammatory
cytokines released
Proliferation
Allo. T cell
mDC
HLA-DR
B7.2
B7.1
CD4
CD1a
Maturation
DC
mDC
Ceramides released
TNF-α
im DC/LC
TNF-α+Seminal plasma
C
Both cascades described
in A and B
No changes in phenotype
and function of DCs

V

EFFECT OF SEMINAL PLASMA ON EXPRESSION OF HIV-1 CO-RECEPTORS AND INFECTION WITH HIV-1

V.1. INTRODUCTION

Heterosexual transmission accounts for most cases of HIV-1 infection (Caceres *et al*, 1996; Mastro *et al*, 1996; Lee *et al*, 1996; AIDS epidemic update, December, 2000, http://www.unaids.org). Evidence that DCs could be involved in HIV transmission was deduced from the observation that HIV readily associates with DCs and caused formation of syncytia *in vitro*, as a result of DC-T cell fusion (Pope *et al*, 1995; Pope *et al*, 1994; Cameron *et al*, 1992). Infection in females may take place primarily in the vagina and cervix, particularly if HIV-susceptible inflammatory cells are present (Levy, 1993; Nuovo *et al*, 1993). The surface of the female genital tract is covered by layers of epithelial cells which provide a protective barrier for the underlying structures. The epithelial cells in the female genital tract are composed of columnar, squamous and transitional cells. The vagina and ectocervix which are most exposed to HIV inoculation are comprised of five distinct cell layers (fig.I.4; Witkin, 1993). In contrast, the endocervix and uterus are composed of a single layer of epithelial cells ('simple epithelium'). HIV-1 susceptible cells, Langerhans' cell, $CD4^+$ T cells and macrophages are predominantly present in parabasal and basal layers of vagina and ectocervix (Johansson *et*

al, 1999; Poppe *et al*, 1998; Morris *et al*, 1983). For sexually transmitted diseases (STDs) including HIV infection, mucosal integrity plays an obvious and important role in transmission. Transmission of HIV would be particularly likely if there are venereal diseases with open lesions and many inflammatory cells present due to other STDs that may provide a portal for HIV entry (Wasserheit, 1992; Dickerson *et al*, 1996; Plummer, 1998; Cohen, 1998; Gray *et al*, 1999).

LCs in the mucosal epithelium may be the first cells to become infected after mucosal exposure to HIV-1 as has been shown in studies using the rhesus macaque model of SIV (Spira *et al*, 1996; Joag *et al*, 1997; Blauvelt *et al*, 1997; Zambruno *et al*, 1995; Miller and Hu, 1999) and using skin explants (Reece *et al*, 1998). It has also been observed that SIV can be efficiently transferred to lymph nodes within two days of vaginal inoculation of the virus in rhesus macaques (Joag *et al*, 1997; Spira *et al*, 1996). HIV-1-carrying DCs migrate to the draining lymph node where interaction with T cells may establish productive infection, and ultimately lead to systemic spread of HIV-1. Animal studies using the SIV-macaque model demonstrated that DC-T cell clusters are a major site of viral replication (Hu *et al*, 1999).

Immature DCs express CCR5, CCR2 and CCR3 (Rubbert *et al*, 1998; Sallusto *et al*, 1998; Sozzani *et al*, 1998[a]; Sallusto and Lanzavecchia, 1999) which are used as co-receptors by R5 strains of HIV-1 although CCR2 and CCR3 usage by this virus is less common than CCR5. More importantly, freshly isolated LCs express functional CCR5 (Zaitseva *et al*, 1997) as well as CD4 (Patterson

et al, 1995, Wright-Browne *et al*, 1997; Lee *et al*, 1999) allowing entry of R5 strains of the virus. Upon maturation of DCs, expression of CXCR4 is induced allowing potential entry of X4 strains of virus (Zaitseva *et al*, 1997; Sallusto *et al*, 1998; Canque *et al*, 1999) and expression of CCR5 is down-regulated (Sallusto *et al*, 1998). Expression of CCR5 and CXCR4 on $CD4^+$ lymphocytes is regulated by cytokines. CCR5 expression on $CD4^+$ cell populations is up-regulated by type 1 cytokines such as IFN-γ and IL-2 and down-regulated by type 2 cytokines such as IL-10 (Patterson *et al*, 1999). Expression of CXCR4 on DCs is up-regulated by IL-4 (Jourdan *et al*, 1998) and TGF-β and down-regulated by IFN-α, IFN-β and IFN-γ (Zoeteweij *et al*, 1998). Therefore, an increase in type 2 cytokine production or lack of type 1 cytokine production, which can occur during HIV infection (Maggi *et al*, 1994; Barcellini *et al*, 1994; Jason *et al*, 1995; Meroni *et al*, 1996; Stylianou *et al*, 1999), may promote infection of DCs with X4 strains of virus. Expression of CXCR4 (Canque *et al*, 1999; Sallusto *et al*, 1998; Zaitseva *et al*, 1997) and CCR7 (Sallusto *et al*, 1999; Dieu-Nosjean *et al*, 1999; Sallusto *et al*, 1998; Yanagihara *et al*, 1998) whose natural ligands (SDF-1 for CXCR4; SLC and ELC for CCR7) are produced in lymphoid organs, is increased on mature DCs, facilitating migration of DCs to the lymph node where DC-T cell interaction occurs. It has been demonstrated that productive infection of DCs with HIV and the ability of DCs to capture virus are mediated through separate pathways (Blauvelt *et al*, 1997). Productive infection of DC is CD4-, CCR5- and CXCR4-dependent, whereas capture of virus is independent of CD4, CCR5 and CXCR4 and is facilitated by the unique dendritic morphology of DCs. A newly identified DC-specific ICAM-3 receptor, DC-SIGN which is

abundantly expressed by DCs present in the mucosa, was also shown to bind to the HIV-1 gp120 and hence to be involved in efficient capture of HIV-1. Furthermore, an interaction between DC-SIGN and ICAM-3 facilitates the infection of T cells (Geijtenbeek *et al*, 2000[b]).

In the previous chapter, the effect of seminal plasma on function and phenotype of non HIV-infected DCs was discussed. This part of thesis describes *in vitro*-infection of DCs with HIV-1, the expression of HIV-1 co-receptors on seminal plasma pulsed-DCs, which may influence infection of the cells with HIV-1 and finally the effect of seminal plasma on *in vitro*-infection of DCs. The allostimulatory capacity of *in vitro*-HIV-1 infected DCs is also described. A pool of seminal plasma as described in II.2.6 was again used to pulse DCs in this chapter. A summary of experiments performed in this part of thesis is illustrated in fig.V.1.

V.2. RESULTS

Data from allogeneic mixed lymphocyte reactions (MLRs) was presented in the form of mean counts per minute (CPM) with standard errors (SE) for triplicates for single representatives and in the form of mean stimulation indices (SI) with SE for summary of all experiments. Data from flow cytometric analyses were presented in the form of mean fluorescence intensity (MFI) with SE. The live MDDC population was gated and designated as R1 (fig.V.2a, 2e, 2i) and expression of HIV-1 co-receptors on MDDCs was analysed within this population. MFI of isotype matched control antibody was

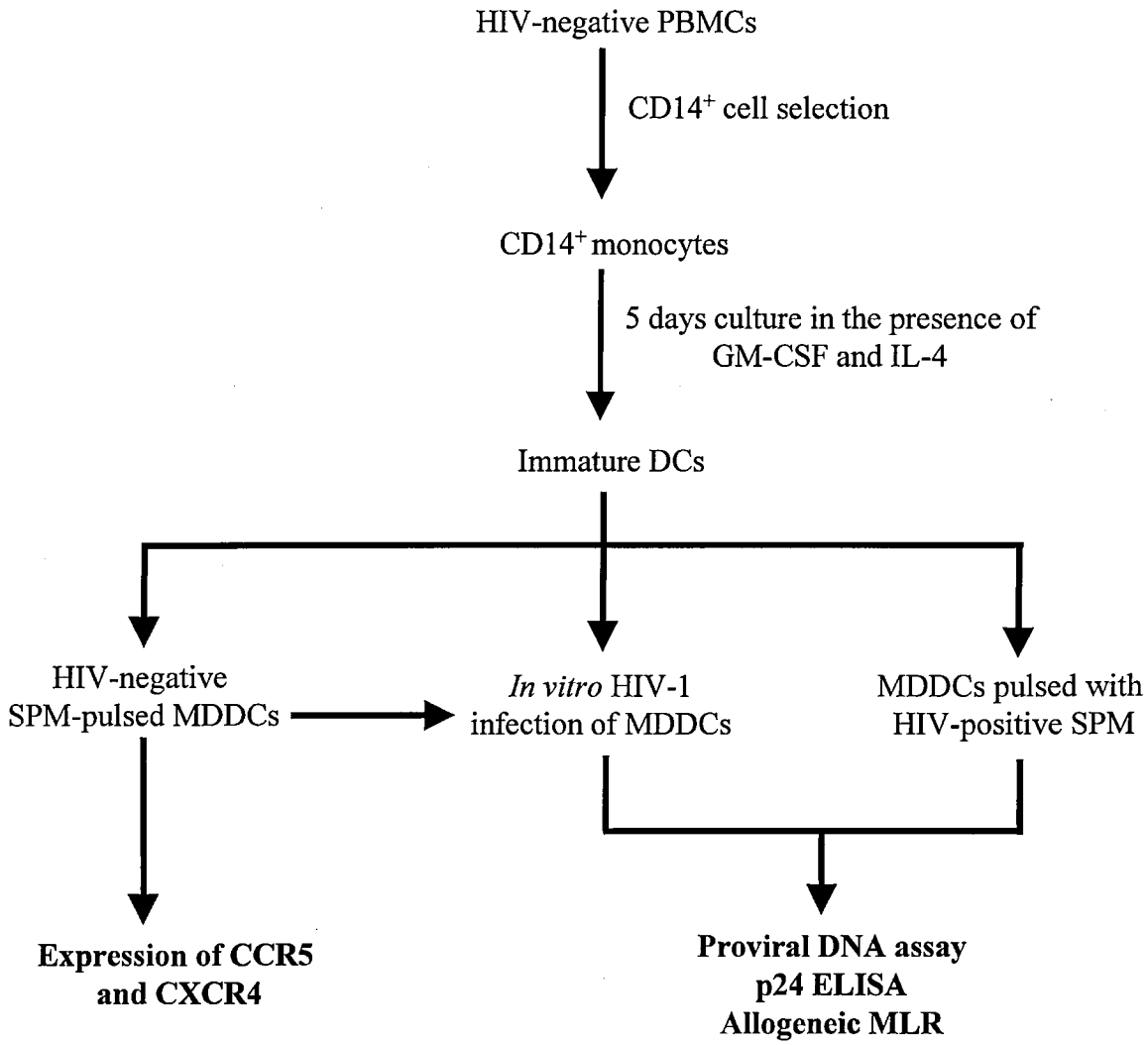


Fig. V.1. Flow chart showing experiments performed in chapter 5

$CD14^+$ monocytes were positively selected from HIV-negative PBMCs using CD14 MicroBeads (II.2.4). $CD14^+$ monocyte-derived DCs (MDDCs) were generated in the presence of GM-CSF and IL-4 (II.2.5). After 5 days of culture cells were pulsed with two different concentrations of HIV-negative seminal plasma (SPM) and/or HIV-1 viral isolates (II.6.3). In some experiments MDDCs were pulsed with HIV-positive SPM. HIV-1 infected MDDCs were assessed for proviral DNA (II.5.2), p24 protein production (II.6.2) and allogeneic capacity (II.2.7). HIV-negative SPM-pulsed DCs were assessed for expression of CCR5 and CXCR4 (II.3.1).

not subtracted from MFI of CCR5 or CXCR4 expression since we are comparing levels of CCR5 or CXCR4 expression on MDDCs which were treated differently (e.g. untreated MDDCs vs. seminal plasma and/or TNF-α-treated MDDCs or between two different concentration of seminal plasma-treated MDDCs). The percentages of positive cells were not assessed since specific antibody binding showed a slight shift compared with isotype matched antibody binding. The degree of increase or decrease in levels of expression was calculated using equation 2 described in II.7. Single representatives of each flow cytometry profile are also shown (fig.V.2a-2c, 2e-2g, 2i-2k).

V.2.1. Surface expression of CCR5 and CXCR4 on $CD14^+$-monocyte-derived DCs (MDDCs)

Whether seminal plasma and/or TNF-α influenced expression of CCR5 and CXCR4 on the MDDC surface was assessed by flow cytometry. MDDCs were derived from HIV-negative PBMCs (II.2.5). MDDCs were pulsed overnight with two different concentrations of seminal plasma pool (II.2.2) and/or 200U/ml (10^6 cells/ml) of TNF-α. MDDCs were washed in 1% FCS/PBS twice before staining. Staining procedures are as described in II.3 except that incubations were at room temperature.

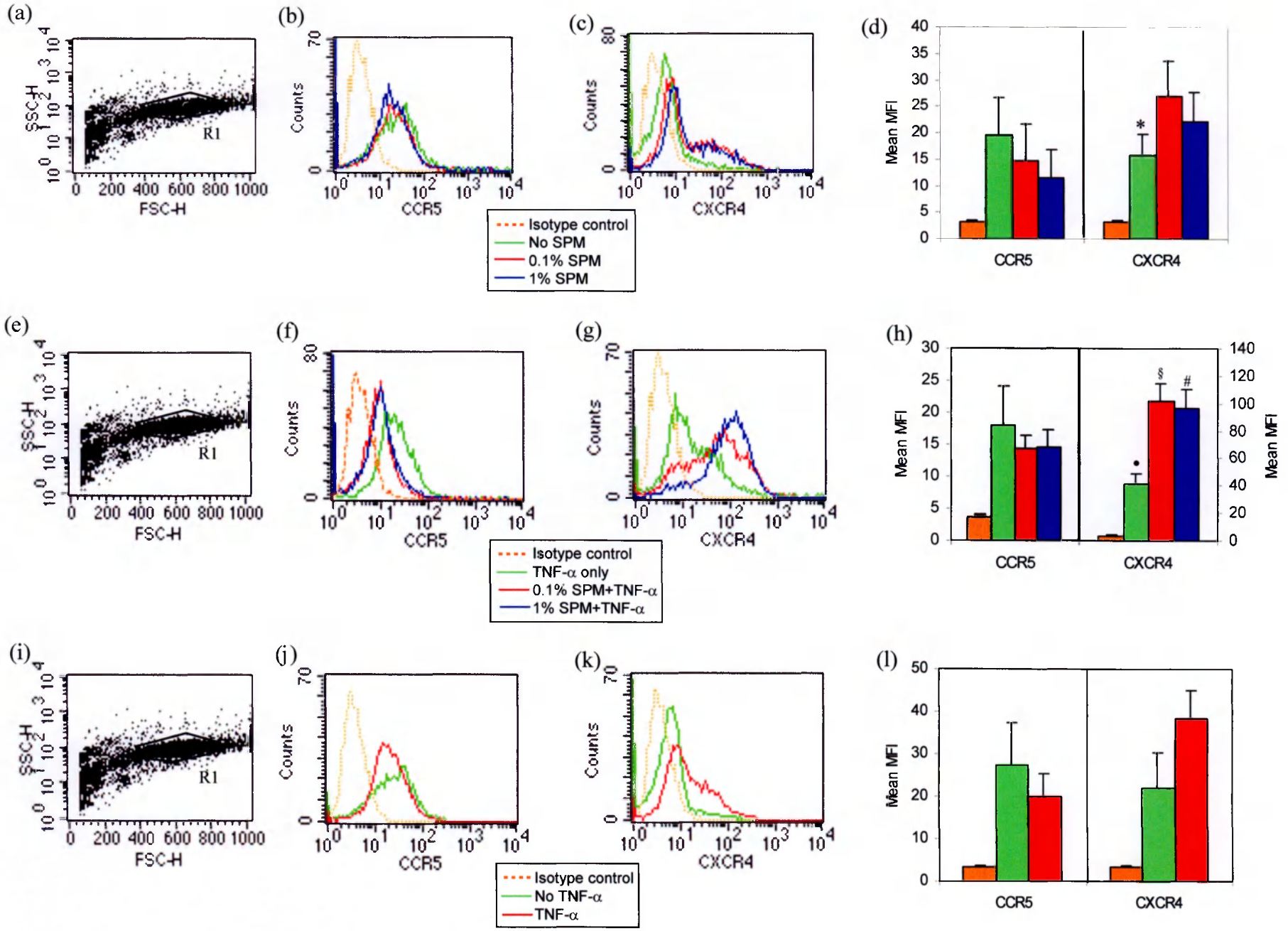
MDDCs expressed both CCR5 and CXCR4 [fig.V.2d, green bars; P=0.06 for CCR5 and P=0.02 for CXCR4 compared with isotype matched control antibody binding (orange bars)]. Expression of CCR5 on MDDCs was decreased by seminal plasma, TNF-α and both seminal plasma and TNF-α although such a decrease was not statistically significant. P values comparing

the level of CCR5 expression on untreated MDDCs and 0.1% or 1% seminal plasma-pulsed MDDCs were >0.05. CCR5 expression was decreased on both 0.1% and 1% seminal plasma-pulsed MDDCs (fig.V.2b and fig.V.2d). The difference in the MFI of CCR5 expression between control MDDCs and 0.1% seminal plasma-pulsed MDDCs was 5.9±2.1 which is 35.3%(±6.7) decrease, and that between control MDDCs and 1% seminal plasma-pulsed MDDCs was 8.9±2.9 which is 50.3%(±8.0) decrease. Similar results were obtained when MDDCs were exposed to both TNF-α and seminal plasma. The level of CCR5 expression was decreased by seminal plasma but not statistically significantly (fig.V.2f and fig.V.2h). TNF-α alone also decreased the level of CCR5 expression on MDDCs when compared with that seen on untreated MDDCs by MFI of 7.4±6.0 (fig.V.2j and fig.V.2i).

Expression of CXCR4 was influenced by seminal plasma or TNF-α to a greater extent than CCR5. Both 0.1% and 1% seminal plasma increased the level of CXCR4 expression on MDDCs by MFI of 11.3±4.5 which is 107.0%(±55.9) increase (P=0.2) and by MFI of 8.0±4.1 which is 73.7%(±50.2) increase (P=0.4) compared with that seen on untreated MDDCs (fig.V.2d). CXCR4 expression appears to be decreased on MDDCs pulsed with 1% seminal plasma compared with that seen on 0.1% seminal plasma-pulsed MDDCs but not significantly (P=0.6; fig.V.2d). Fig.V.2g and fig.V.2h show that MDDCs exposed to both TNF-α and seminal plasma display significantly increased expression of CXCR4. The differences in MFI of CXCR4 between MDDCs exposed to TNF-α and MDDCs exposed to both TNF-α and 0.1% or 1% seminal plasma were 61.0±11.2 [166.6%(±37.0) increase; P=0.006] and

Fig.V.2. Expression of CCR5 and CXCR4 on HIV-negative seminal plasma-pulsed MDDCs.

MDDCs were pulsed with two different concentrations (0.1%, 1%) of HIV-negative SPM or TNF-α or both overnight. Cells were washed next day and stained with anti-CCR5 antibody or anti-CXCR4 antibody as described in II.3.1. The graphs show expression of CCR5 or CXCR4 in MDDC population (R1 in fig.V.3a, V.3e, V.3i) which was pulsed with seminal plasma (a)-(d), was pulsed with both seminal plasma and TNF-α (e)-(h), and was pulsed with TNF-α only (i)-(l). Expression of HIV co-receptors on MDDCs was analysed within this population. (a), (e), and (i): single representatives of dot plot showing a MDDC population; (b), (f), and (j): single representatives of histogram showing expression of CCR5; (c), (g), and (k): single representatives of histogram showing expression of CXCR4; (d), (h), and (l): mean fluorescence intensities (MFI) of CCR5 or CXCR4 expression in a MDDC population with standard errors for 8 and 7 experiments for CCR5 and CXCR4, respectively (d), for 5 experiments for both CCR5 and CXCR4 [(h) and (i)]. P=0.02 (*) comparing MFI of isotype matched control antibody with MFI of CXCR4 expressed on control MDDCs; P=0.02 (•) comparing MFI of isotype matched control antibody with MFI of CXCR4 expressed on TNF-α-pulsed MDDCs; P=0.006 (§) comparing MFI of CXCR4 expressed on TNF-α-pulsed MDDCs with that on MDDCs exposed to both TNF-α and 0.1% seminal plasma; P=0.02 (#) comparing MFI of CXCR4 expressed on TNF-α-pulsed MDDCs with that on MDDCs exposed to both TNF-α and 1% seminal plasma. Statistical method employed was 'independent-sample T test' using SPSS 10.0 software.


(a)
(b)
(c)
(d)
SSC-H
FSC-H
R1
Counts
CCR5
CXCR4
Isotype control
No SPM
0.1% SPM
1% SPM
Mean MFI
(e)
(f)
(g)
(h)
TNF-α only
0.1% SPM+TNF-α
1% SPM+TNF-α
(i)
(j)
(k)
(l)
No TNF-α
TNF-α

55.7±21.51 [82.9%(±84.5) increase; P=0.02], respectively. TNF-α alone also increased the level of CXCR4 expression on MDDCs by MFI of 16.3±2.2, 142.2%(±46.8) increase, although such an increase was not statistically significant (fig.V.2k and fig.V.2l; P=0.2).

V.2.2. Assessment of MDDC infection with HIV-1

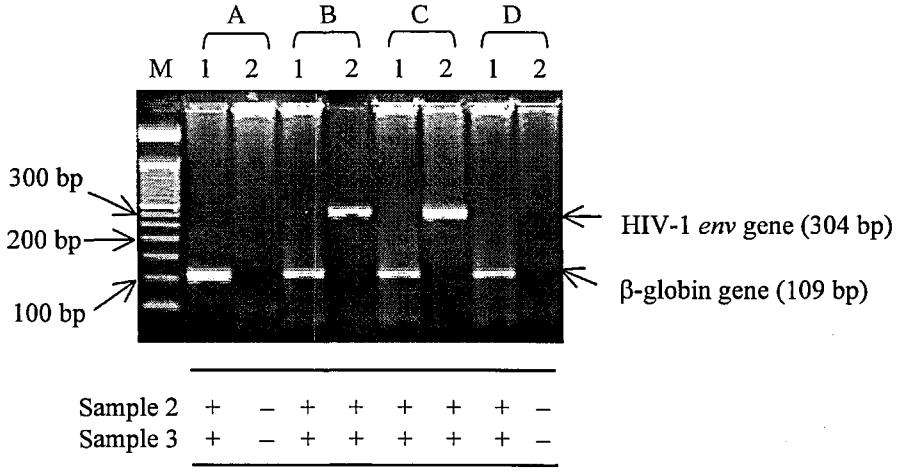
HIV-negative seminal plasma pulsed-MDDCs and control MDDCs (i.e. untreated MDDCs) were exposed to different strains of HIV (table II.2) as described in II.6.3. Infection of MDDCs with HIV-1 was assessed using two different methods: i) a viral DNA assay using HIV-1 specific *env* primers and β-globin primers as a positive control, and ii) a p24 protein ELISA for measurement of p24 antigen levels in culture supernatants (frozen and thawed).

Fig.V.3 shows that MDDCs could be infected with virus *in vitro*. Prior to adding to MDDC culture the virus was treated with DNase and this removed DNA from the viral stock as demonstrated by viral DNA assay (fig.V.3d). Amplification of HIV *env* DNA demonstrated that without seminal plasma (Lane B) MDDCs were infected with JRCSF virus [R5 strain (fig.V.3a] and with evidence of a much lower level of infection with PE106 virus [R5/X4 strain (fig.V.3b)]. Infection with JW5 virus [X4 strain (fig.V.3c)] was not observed. Infection with JRCSF virus was also detected in MDDCs pulsed with 0.1% seminal plasma (fig.V.3a, Lane C) but not in MDDCs pulsed with 1% seminal plasma (fig.V.3a, Lane D). When MDDCs were pulsed with 1% seminal plasma the level of CCR5 expression decreased, as previously

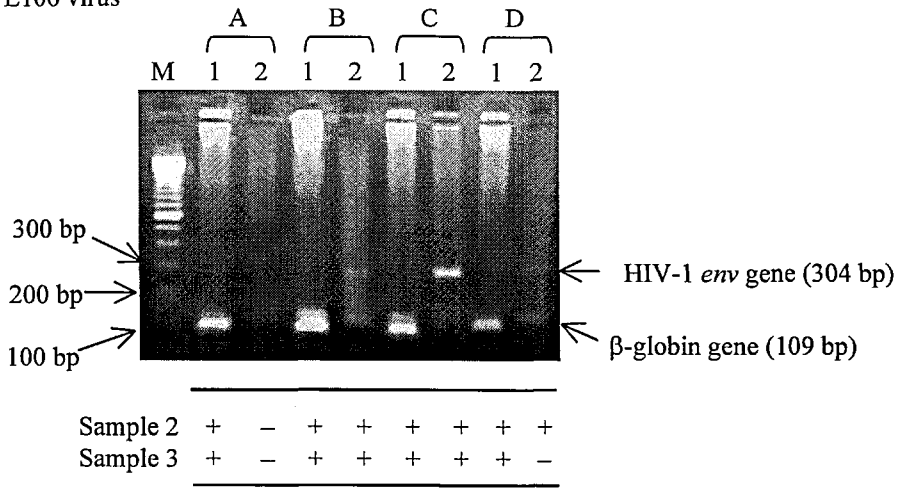
Fig. V.3. Viral DNA detection in MDDCs infected with HIV-1 *in vitro*.

0.1% or 1% seminal plasma together with p24 concentration of 0.1ng/ml of JRCSF (a), PE106 (b) or JW5 (c) viruses were added to MDDC cultures. Virus was pre-treated with DNase to degrade DNA if present in the virus stocks. Viral DNA (*env*) was not detected in DNase treated virus (d). OM10.1 cells were used as a positive control. Following exposure cells were washed three times and DNA was extracted next day using Tri Reagent™: Uninfected MDDCs (Lane A), MDDCs infected with virus without seminal plasma (Lane B), MDDCs infected with virus in the presence of 0.1% seminal plasma (Lane C) and MDDCs infected with virus in the presence of 1% seminal plasma (Lane D). Viral DNA was assessed by a nested PCR and visualised on 1.5% agarose gel in the presence of ethidium bromide. Lane 1 represents β-globin gene amplification (109 bp) and lane 2 represents HIV-1 *env* gene amplification (304 bp). Marker (M) used in (a) is 50 bp and (b), (c) and (d) is 100 bp. Figure shows a representative of 3 experiments (sample 1).

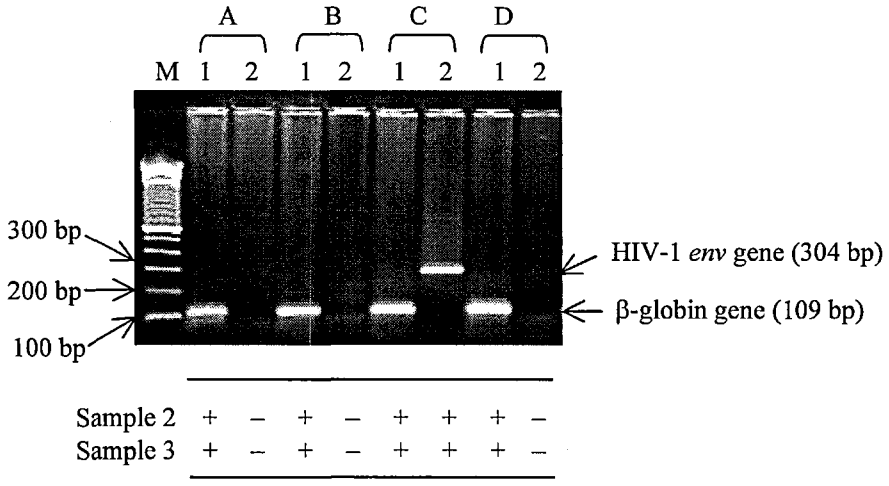
(a) JRCSF virus



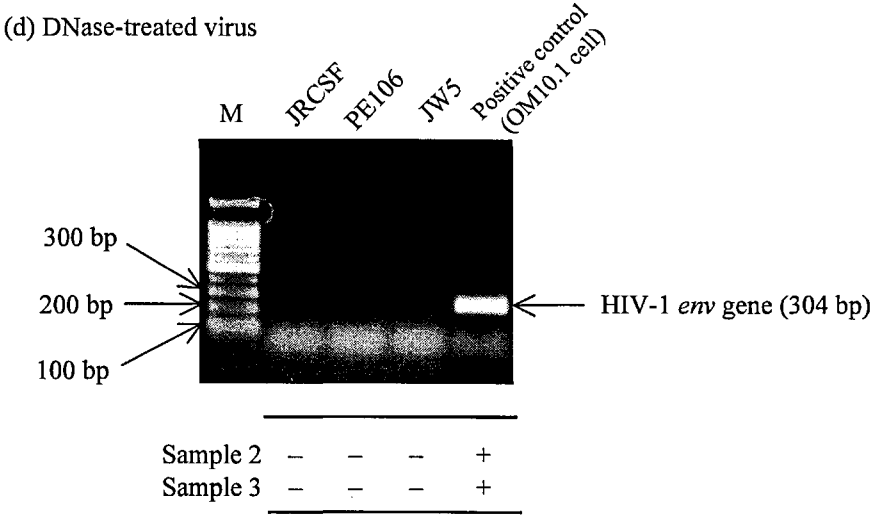
(b) PE106 virus



(c) JW5 virus



(d) DNase-treated virus



described (fig.V.2d). The viral DNA assay demonstrated that JRCSF virus was unable to infect 1% seminal plasma-pulsed MDDCs and this may be due to reduced levels of CCR5 expression on MDDCs. Infection with PE106 virus was greatly enhanced when 0.1% seminal plasma was added (fig.V.3b, Lane C) and was decreased when 1% seminal plasma was added (fig.V.3b, Lane D) to the culture. Detectable levels of infection with JW5 virus were observed only when 0.1% seminal plasma was present in the culture (fig.V.3c; Lane C). Although MDDCs expressed CXCR4 (fig.V.2d) the level of CXCR4 expression on these untreated MDDCs may not be sufficient for entry of X4 strain of virus. However, 0.1% seminal plasma pulsed MDDCs displayed increased levels of CXCR4 expression, which may have enhanced entry of X4 strain of virus. Infections with PE106 virus and JW5 virus were not detected in MDDCs pulsed with 1% seminal plasma (fig.V.3b and fig.V.3c). Although statistical analyses on the level of CXCR4 expression on 1% seminal plasma-pulsed MDDCs showed no significant difference from 0.1% seminal plasma-pulsed MDDCs, failure in infecting 1% seminal plasma-pulsed MDDCs suggests that the level of CXCR4 expression on these cells may not be sufficient for entry of X4 strains of virus as seen in control MDDCs (the level of CXCR4 expression on 1% seminal plasma-pulsed MDDCs was not significantly different from control MDDCs).

p24 levels in supernatants of HIV-1 infected MDDC cultures which were collected at different time points during the cultivation, were measured by ELISA. p24 protein in the supernatants was present at a very low concentration or below the detection limit (table V.1). For MDDC sample 1 it

Table V.1. p24 protein production by MDDCs infected with HIV-1 *in vitro*

Seminal plasma and HIV-1 (final concentration of 0.1ng/ml) were added to MDDCs, cells were washed three times and resuspended in 1ml of DC medium. Levels of p24 protein in the supernatants were collected at different time points and were measured by ELISA (II.6.2). (LDL=Lower than Detection Limit; detection limit=1ng/ml)

Sample description			p24 protein concentration (ng/ml)
Sample 1	JRCSF only	Day 2	LDL
		Day 5	LDL
		Day 6	LDL
	JRCSF+0.1% seminal plasma	Day 2	LDL
		Day 5	1.25
		Day 6	1.28
	JRCSF+1% seminal plasma	Day 2	LDL
		Day 5	LDL
		Day 6	LDL
Sample 2	PE106 only	Day 5	1.4
	PE106+0.1% seminal plasma	Day 5	LDL
	PE106+1% seminal plasma	Day 5	LDL
Sample 3	JW5 only	Day 5	LDL
	JW5+0.1% seminal plasma	Day 5	1.0
	JW5+1% seminal plasma	Day 5	LDL

appears that a lower concentration of seminal plasma (0.1%) enhanced infection of MDDCs with R5 strains of HIV-1 (day 5 and day 6), however it was not the case for MDDC sample 2 and 3. This data suggests that productive infection of these cells with HIV-1 in this system was not efficient enough to release measurable amount of p24 by ELISA or our ELISA system is not sensitive enough. Therefore, supernatants from the allogeneic MLR using HIV-1 infected MDDCs were collected and p24 levels in the supernatants were measured by ELISA. Amounts of p24 protein in the allogeneic MLR supernatants were still lower than detection limit, 0.0316ng/ml (table V.2).

Virus in seminal plasma from HIV^+ individuals was capable of infecting MDDCs since viral DNA was detected in cells which were incubated with seminal plasma from HIV^+ patients (fig.V.4). Fig.V.4a shows viral DNA detection in MDDCs pulsed overnight with seminal plasma from a HIV^+ patient whose viral load in seminal plasma was 2,000 copies/ml (patient 9; refer table III.1 in chapter III for patient details). Fig.V.4b shows viral DNA detection in MDDCs pulsed with seminal plasma from a HIV^+ patient whose viral load in seminal plasma was 68,000 copies/ml (patient 10; refer table III.1 in chapter III for patient details). When MDDCs were pulsed with seminal plasma with a viral load of 2,000 copies/ml, viral DNA was detected in cells pulsed with 1% seminal plasma (equivalent to 20 copies of virus), but not in cells pulsed with 0.1% seminal plasma (equivalent to 2 copies of virus) (fig.V.4a). In contrast, when seminal plasma which has a higher viral load (68,000 copies/ml) was used to pulse MDDCs viral DNA was detected in MDDC populations pulsed with both 0.1% (equivalent to 68 copies of virus)

Table V.2. p24 protein production by allogeneic MLRs using MDDCs infected with HIV-1 *in vitro*

Final concentrations of 0.1ng/ml of virus and seminal plasma (0%, 0.1%, 1%) were added to MDDC cultures. Cells were washed three times and an allogeneic MLR was set up (10^5 responder cells/well) next day. At day 4, supernatants were collected and p24 protein concentrations were measured by ELISA (II.6.2). (LDL=Lower than Detection Limit; detection limit=0.0316ng/ml)

Sample description \ DC No. per well		125	250	500	1000	2000	4000	8000
Sample 1	PE106 only	LDL	LDL	LDL	LDL	LDL	0.2	LDL
	PE106+0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	PE106+1% seminal plasma	0.12	LDL	0.14	LDL	LDL	LDL	LDL
Sample 2	JW5 only	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JW5+0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JW5+1% seminal plasma	LDL	~0.1	LDL	LDL	0.18	0.316	LDL
Sample 3	JRCSF only	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JRCSF+0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JRCSF+1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	PE106 only	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	PE106+0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	PE106+1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JW5 only	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JW5+0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
	JW5+1% seminal plasma	0.1	LDL	LDL	LDL	LDL	LDL	LDL

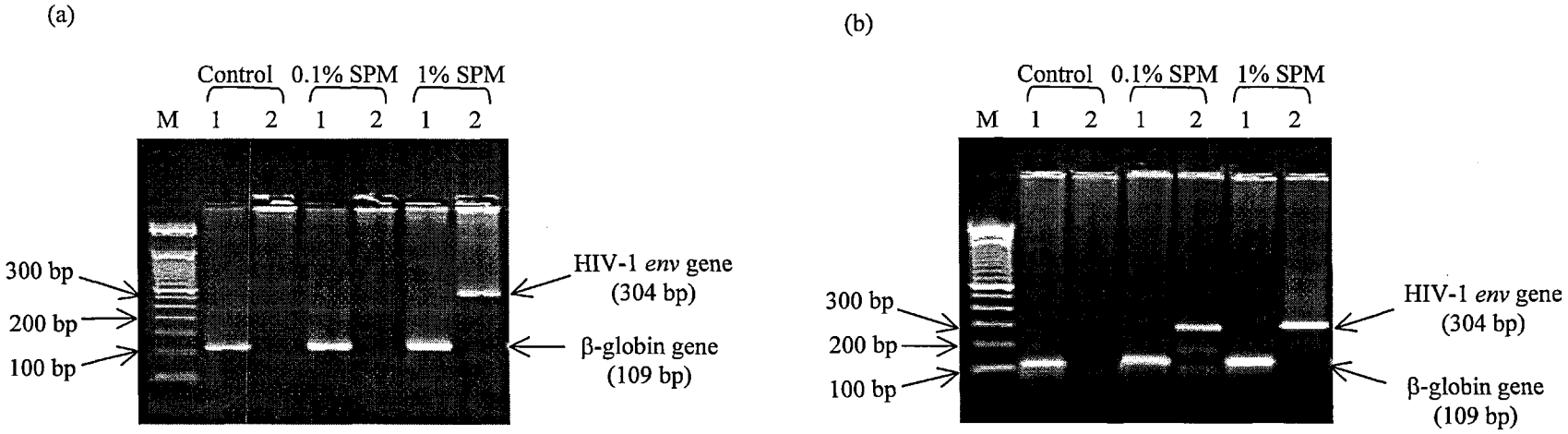


Fig. V.4. Viral DNA assay by nested PCR

HIV-negative MDDCs were incubated with 0.1% or 1% seminal plasma (SPM) from HIV^+ patients whose viral load in SPM was 2,000 copies/ml (a) or 68,000 copies/ml (b) overnight. DNA from the cells was extracted after further 2 days of incubation, using Tri-reagent™. Viral DNA was assessed by a nested PCR and visualised on 1.5% agarose gel in the presence of ethidium bromide. Lane 1 represents β-globin gene amplification (109 bp) and lane 2 represents HIV-1 *env* gene amplification (304 bp). Marker (M) used in (a) is 50 bp and in (b) is 100 bp.

and 1% (equivalent to 680 copies of virus) seminal plasma (fig.V.4b). This data suggests that viral strains present in seminal plasma can infect DCs efficiently. Final viral concentrations of as little as 20 copies/ml was sufficient to infect these cells and the infection could be detected by a nested PCR. An allogeneic MLR was set up using MDDCs that were pulsed with seminal plasma from patient 10. Despite the fact that viral DNA was detected in MDDCs which were *in vitro*-infected with virus present in seminal plasma, levels of p24 in supernatants of allogeneic MLR were lower than detection limit (0.0316ng/ml; table V.3). This data suggests that HIV enters MDDCs, with reverse transcription of viral RNA to DNA (as demonstrated by PCR; fig.V.4) but with little or no productive replication of virus and hence may not have transmitted virus to allogeneic T cells.

V.2.3. Assessment of antigen presenting function of HIV-1 infected $CD14^+$-monocyte-derived DCs (MDDCs)

Antigen presenting function of MDDCs which were infected with virus was assessed by allogeneic MLRs. After 5 days of culture MDDCs were pulsed with virus exhibiting different cellular tropism (JRCSF: CCR5-utilising strain; PE106: CCR5 and CXCR4-utilising strain; JW5: CXCR4-utilising strain) overnight. Cells were washed and were used to stimulate allogeneic T cells. Cells were cultured for further 4 days. The data showed that infection with virus, as confirmed by PCR (fig.V.3) enhanced allostimulatory potentials of MDDCs but not significantly (fig.V.5). It appears that JW5 virus has the least effect on the allostimulatory potentials of MDDCs (fig.V.5). This may be associated with the level of infection of MDDCs. Viral DNA assay

Table V.3. p24 protein production by allogeneic MLR using MDDCs exposed to HIV-1$^+$ seminal plasma

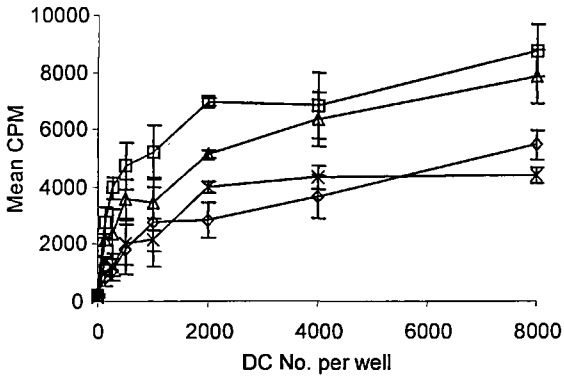
Seminal plasma (0%, 0.1%, 1%) from patient 10 (HIV-1 viral load in seminal plasma was 68,000 copies /ml) was added to MDDC cultur. Cells were washed three times and an allogeneic MLR was set up (10^5 responder cells/well) next day. At day 4, supernatants were collected and p24 protein concentration was measured by ELISA (II.6.2) (LDL=Lower than Detection Limit; detection limit is 0.0316ng/ml)

DC No. per well / Sample description	125	250	500	1000	2000	4000	8000
0.1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL
1% seminal plasma	LDL	LDL	LDL	LDL	LDL	LDL	LDL

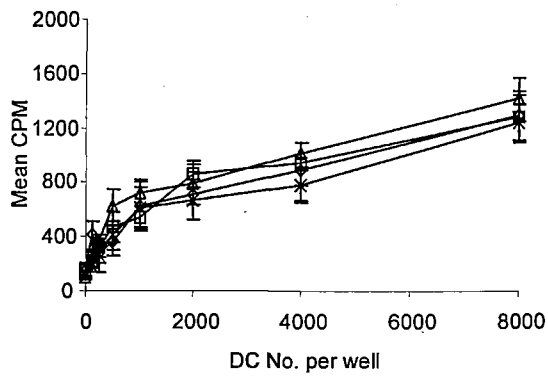
Fig. V.5. Allogeneic MLR with MDDCs pulsed with virus with different cellular tropism.

MDDCs were pulsed with virus exhibiting different cellular tropism (JRCSF: CCR5-utilising strain; PE106:CCR5 and CXCR4-utilising strain; JW5:CXCR4-utilising strain) overnight. Cells were washed and an allogeneic MLR was set up next day using 10^5 responder cells per well. Cells were cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs (a), (b), and (c) show mean count per minute (CPM) with standard errors of each experiment. Graph (d) shows mean stimulation index (SI) of 3 experiments with standard errors. Each experiment was set up in triplicate. The table below the graphs shows P values comparing ability of MDDCs which were exposed to virus to stimulate allogeneic T cells with that of control MDDCs. P values comparing allostimulatory ability of DCs exposed to different strains of virus are also shown. P values were evaluated from mean SI of 3 experiments and standard errors. Statistical analysis method employed was 'independent sample T test' and performed uing a SPSS 10.0 software. SI was calculated by dividing mean CPM of background (ie. no DC) from mean CPM of each DC number (equation 1 in II.7).

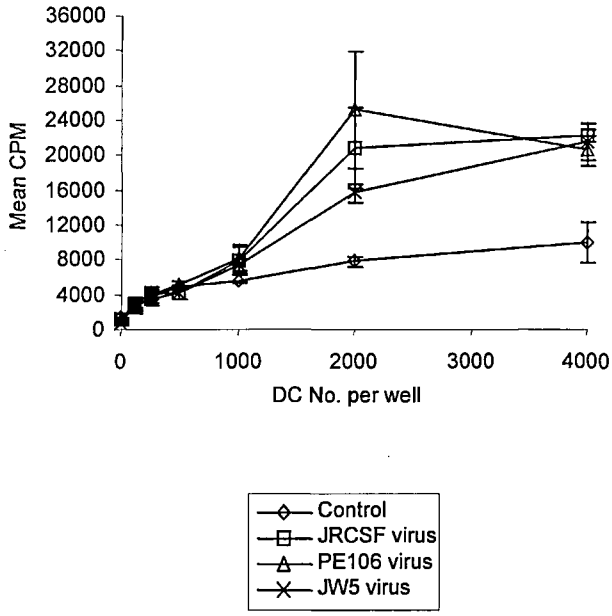
(a)



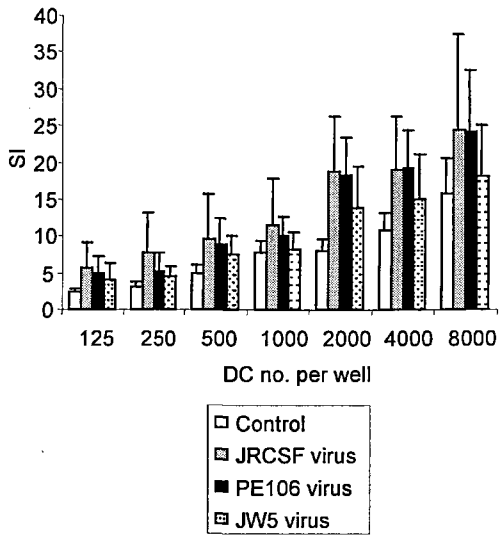
(b)



(c)



(d)



DC No. per well		125	250	500	1000	2000	4000	8000
P values (vs. control)	JRCSF	0.4	0.4	0.4	0.5	0.2	0.2	0.6
	PE106	0.3	0.4	0.2	0.1	0.1	0.1	0.3
	JW5	0.5	0.4	0.3	0.7	0.3	0.4	0.6
P values (vs. JRCSF virus)	PE106	0.7	0.4	0.9	0.7	0.9	0.9	0.9
	JW5	0.4	0.5	0.6	0.5	0.2	0.2	0.5
P values (vs. PE106 virus)	JW5	0.01	0.7	0.5	0.09	0.4	0.1	0.1

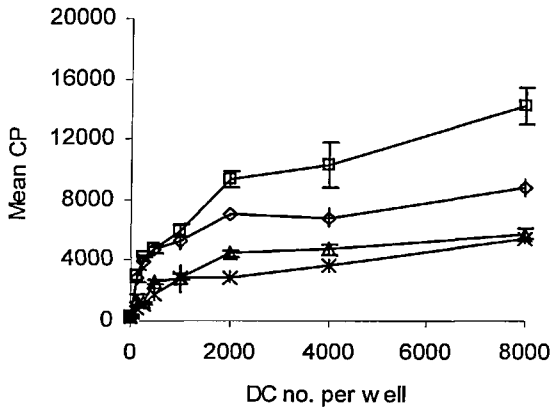
demonstrated undetectable level of JW5 virus DNA by PCR (fig.V.3c, lane B). It was expected that antigen-carrying DCs would undergo maturation and hence their allostimulatory function would be enhanced. Therefore, it is not surprising that JW5 virus exposed-MDDCs which did not have detectable levels of viral DNA, displayed allostimulatory potentials similar to those of control MDDCs (i.e. unexposed to virus; fig.V.5).

Antigen presenting function of MDDCs exposed to both virus and seminal plasma was assessed to determine whether presence of virus abolished the suppressed allostimulatory capacity of seminal plasma-pulsed MDDCs (chapter IV) or whether seminal plasma altered the allostimulatory potentials of antigen-carrying DCs. First of all, two experiments demonstrated a significantly increased allostimulatory ability of JRCSF virus-infected MDDCs when compared with that of uninfected control MDDCs (fig.V.6a and fig.V.6c, P=0.000 and P=0.001, respectively). These two experiments also demonstrated a decreased allostimulatory ability of MDDCs when exposed to JRCSF virus in the presence of 1% seminal plasma compared with that of MDDCs exposed to virus only (P=0.007 for fig.V.6a and P=0.001 for fig.V.6c). When the allostimulatory potential of MDDCs exposed to both JRCSF virus and 1% seminal plasma was compared with that of control MDDCs (i.e. not exposed to either seminal plasma or virus) the suppressive effect of seminal plasma was not observed (fig.V.6a and fig.V.6c, P=0.8 and P=0.06, respectively). The summary of all experiments showed a tendency of increased allostimulatory potentials when MDDCs were exposed to virus (without seminal plasma and with 0.1% seminal plasma) although it was not

Fig. V.6. Allogeneic MLR with MDDCs infected with JRCSF virus and/or pulsed with different concentrations of HIV-negative seminal plasma

MDDCs were pulsed with JRCSF virus (CCR5-utilising strain) alone or together with 0.1% or 1% HIV-negative seminal plasma overnight. Cells were washed and allogeneic MLRs were set up next day using 10^5 responder cells per well. Cells were cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs (a), (b), and (c) show mean counts per minute (CPM) with standard errors. Graphs (d) shows mean stimulation index (SI) of 3 experiments with standard errors. Each experiment was set up in triplicate. The table below graph (d) shows P values comparing ability of HIV-1 infected MDDCs which were exposed to 0.1% or 1% seminal plasma to stimulate allogeneic T cells with that of HIV-1 infected MDDCs which were not exposed to seminal plasma or with that of uninfected MDDCs. Also, P values comparing allostimulatory ability of MDDCs exposed to different concentrations of seminal plasma were shown. Statistical analyses method employed was 'Independent sample T test' and were performed using a SPSS 10.0 software. SI was calculated by dividing mean CPM of background (ie. no MDDC) from mean CPM of each MDDC number (equation 1 described in II.7). Refer the text for statistical analyses on individual experiment presented in fig.V.6a-fig.V.6c.

(a)



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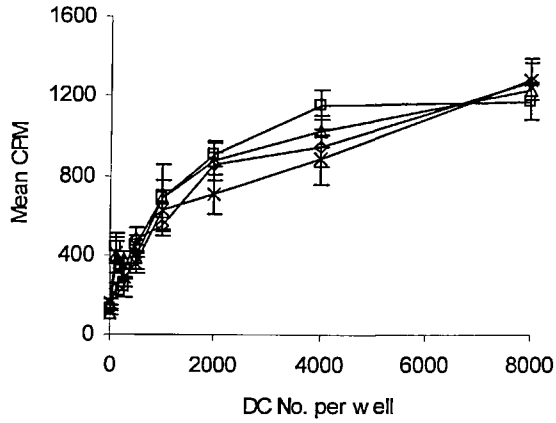

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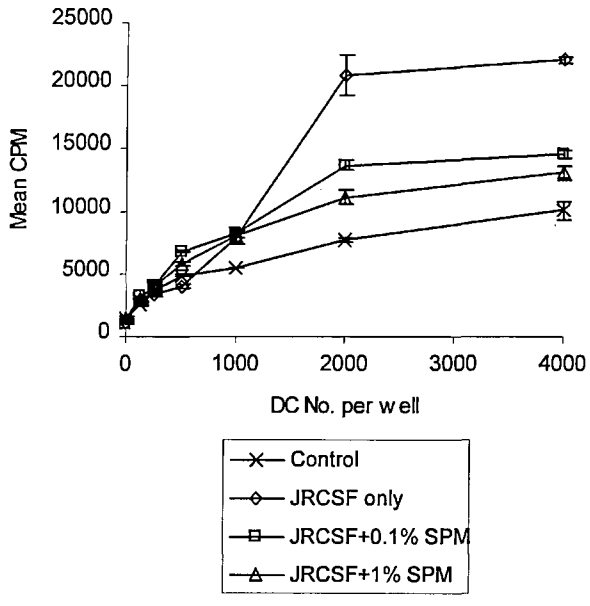
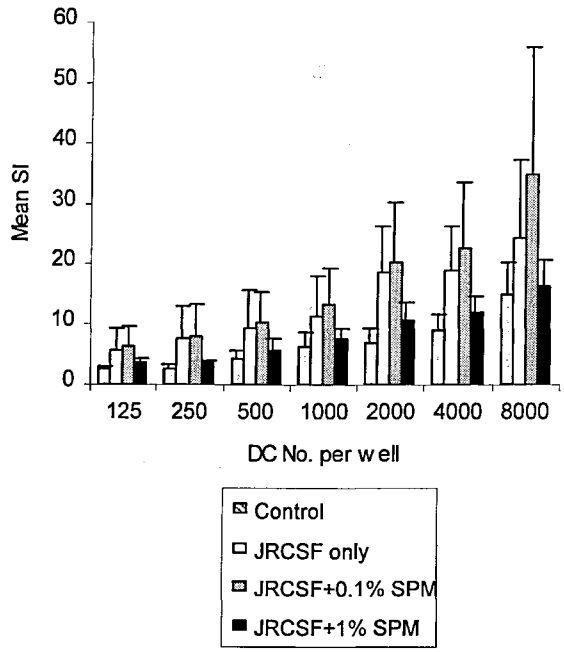


(d)



DC No. per well		125	250	500	1000	2000	4000	8000
P values (vs. control)	JRCSF only	0.5	0.5	0.5	0.5	0.2	0.3	0.6
	JRCSF+0.1% SPM	0.3	0.4	0.3	0.4	0.3	0.3	0.5
	JRCSF+1% SPM	0.3	0.5	0.6	0.7	0.4	0.5	0.9
P values (vs. JRCSF only)	JRCSF+0.1% SPM	0.8	1.0	0.9	0.9	0.9	0.8	0.8
	JRCSF+1% SPM	0.6	0.5	0.6	0.6	0.4	0.4	0.7
P values (vs. JRCSF+0.1% SPM)	JRCSF+1% SPM	0.4	0.5	0.5	0.4	0.4	0.4	0.6

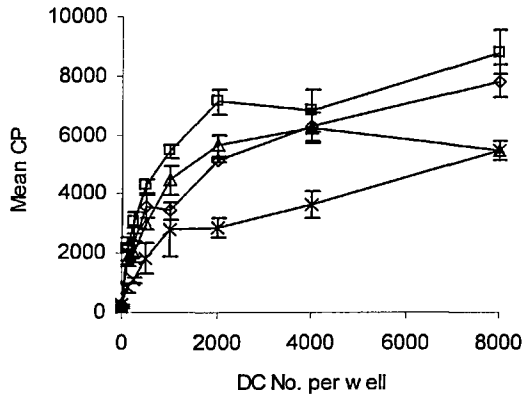
statistically significant (fig.V.6d). Neither such increases nor significant suppression in the allostimulatory capacity of MDDCs were not seen when cells were exposed to both 1% seminal plasma and virus when compared with control MDDCs (fig.V.6d). These data were correlated with the data obtained from viral DNA assay (fig.V.3a) which showed detectable level of viral DNA in both MDDCs exposed to virus only and MDDCs exposed to virus together with 0.1% seminal plasma, but not in MDDCs exposed to virus and 1% seminal plasma. Taken together the data suggests that allostimulatory potentials of MDDCs which are infected with HIV were related to the level of infection with HIV. Although MDDCs exposed to JRCSF virus in the presence of 1% seminal plasma did not have detectable level of viral DNA these cells did not show the seminal plasma-induced suppressed allostimulatory capacity which was previously seen in chapter IV. This may be due to infection of allogeneic T cells with HIV which were captured by or attached to MDDCs. This can be possible as MDDCs has 'dendritic' morphology and also 1% seminal plasma-pulsed MDDCs express CD4 (fig.IV.4 in chapter IV).

Similar results were obtained when PE106 virus infected MDDCs or JW5 virus infected MDDCs were used (fig.V.7 and fig.V.8). Two experiments showed a significant increase in allostimulatory capacity of MDDCs infected with PE106 virus compared with that of control MDDCs (fig.V.7a and fig.V.7b, P=0.01 and P=0.000, respectively). All three experiments demonstrated highest allostimulatory potential of MDDCs which were infected with PE106 in the presence of 0.1% seminal plasma. This was, again, correlated with data obtained from viral DNA assay showing high level of

Fig. V.7. Allogeneic MLR with MDDCs infected with PE106 virus and/or pulsed with different concentrations of HIV-negative seminal plasma

MDDCs were pulsed with PE106 virus (both CCR5 and CXCR4-utilising strain) alone or together with 0.1% or 1% HIV-negative seminal plasma overnight. Cells were washed and allogeneic MLRs were set up next day using 10^5 responder cells per well. Cells were cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs (a), (b), and (c) show mean counts per minute (CPM) with standard errors. Graphs (d) shows mean stimulation index (SI) of 3 experiments with standard errors. Each experiment was set up in triplicate. The table below graph (d) shows P values comparing ability of HIV-1 infected MDDCs which were exposed to 0.1% or 1% seminal plasma to stimulate allogeneic T cells with that of HIV-1 infected MDDCs which were not exposed to seminal plasma or with that of uninfected MDDCs. Also, P values comparing allostimulatory ability of MDDCs exposed to different concentrations of seminal plasma were shown. Statistical analyses method employed was 'Independent sample T test' and were performed using a SPSS 10.0 software. SI was calculated by dividing mean CPM of background (ie. no MDDC) from mean CPM of each MDDC number (equation 1 described in II.7). Refer the text for statistical analyses on individual experiment presented in fig.V.7a-fig.V.7c.

(a)



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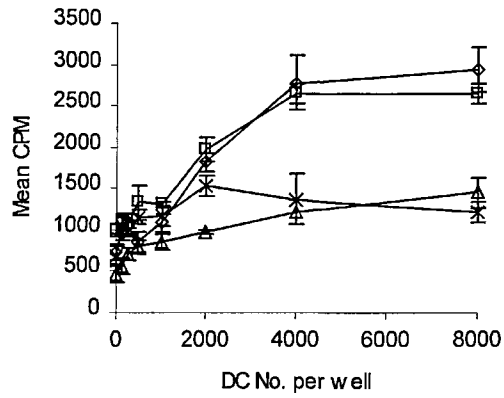

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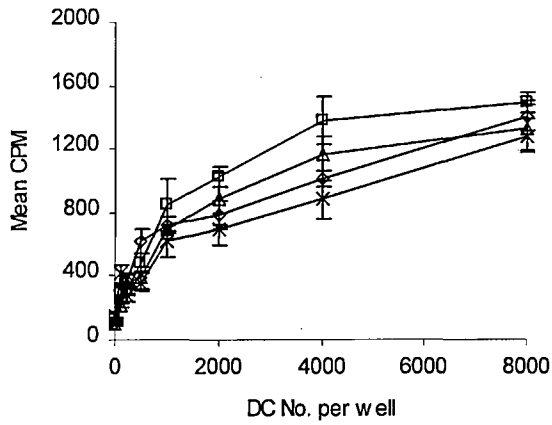

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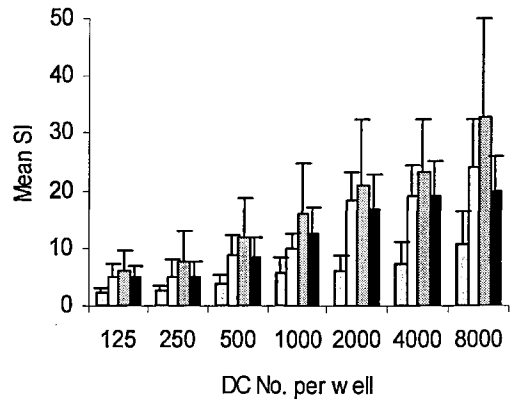
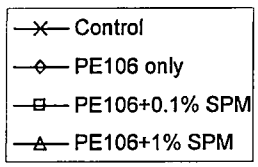
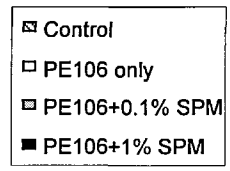




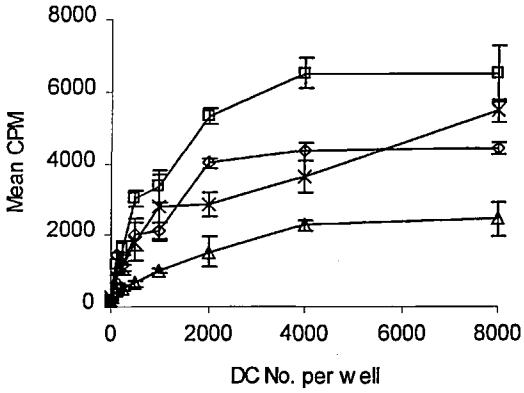


		125	250	500	1000	2000	4000	8000
P values (vs. control)	PE106 only	0.4	0.4	0.3	0.3	0.1	0.1	0.3
	PE106+0.1% SPM	0.4	0.5	0.4	0.3	0.3	0.2	0.5
	PE106+1% SPM	0.3	0.4	0.3	0.3	0.2	0.2	0.4
P values (vs. PE106 only)	PE106+0.1% SPM	0.8	0.7	0.7	0.5	0.8	0.7	0.7
	PE106 +1% SPM	0.9	1.0	0.9	0.7	0.9	1.0	0.8
P values (vs. PE106+0.1% SPM)	PE106 +1% SPM	0.8	0.7	0.7	0.7	0.8	0.7	0.6

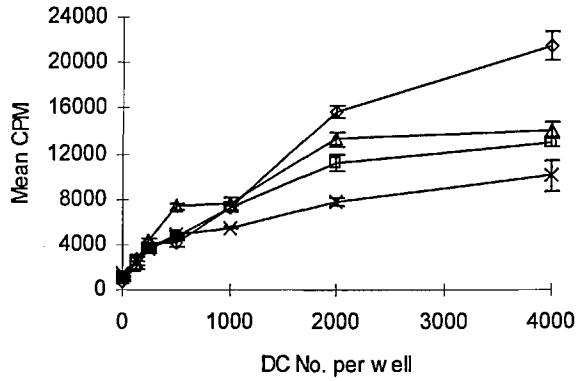
Fig. V.8. Allogeneic MLR with MDDCs infected with JW5 virus and/or pulsed with different concentrations of HIV-negative seminal plasma

MDDCs were pulsed with JW5 virus (CXCR4-utilising strain) alone or together with 0.1% or 1% HIV-negative seminal plasma overnight. Cells were washed and allogeneic MLRs were set up next day using 10^5 responder cells per well. Cells were cultured for further 4 days. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well at day 4 and harvested at day 5. Graphs (a), (b), and (c) show mean counts per minute (CPM) with standard errors. Graphs (d) shows mean stimulation index (SI) of 3 experiments with standard errors. Each experiment was set up in triplicate. The table below graph (d) shows P values comparing ability of HIV-1 infected MDDCs which were exposed to 0.1% or 1% seminal plasma to stimulate allogeneic T cells with that of HIV-1 infected MDDCs which were not exposed to seminal plasma or with that of uninfected MDDCs. Also, P values comparing allostimulatory ability of MDDCs exposed to different concentrations of seminal plasma were shown. Statistical analyses method employed was 'Independent sample T test' and were performed using a SPSS 10.0 software. SI was calculated by dividing mean CPM of background (ie. no MDDC) from mean CPM of each MDDC number (equation 1 described in II.7). Refer the text for statistical analyses on individual experiment presented in fig.V.8a-fig.V.8c.

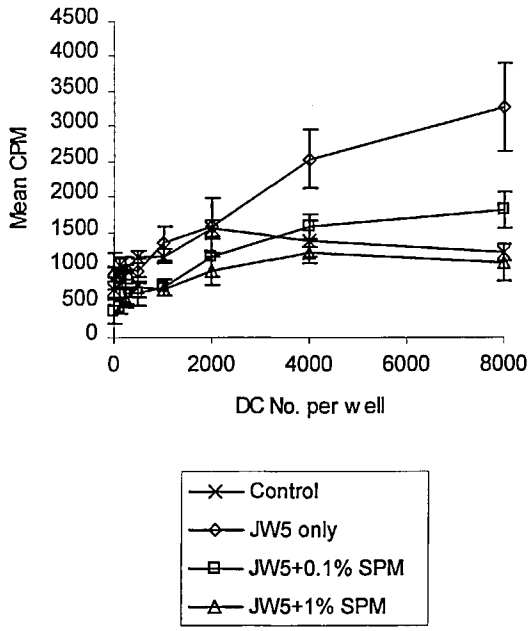
(a)



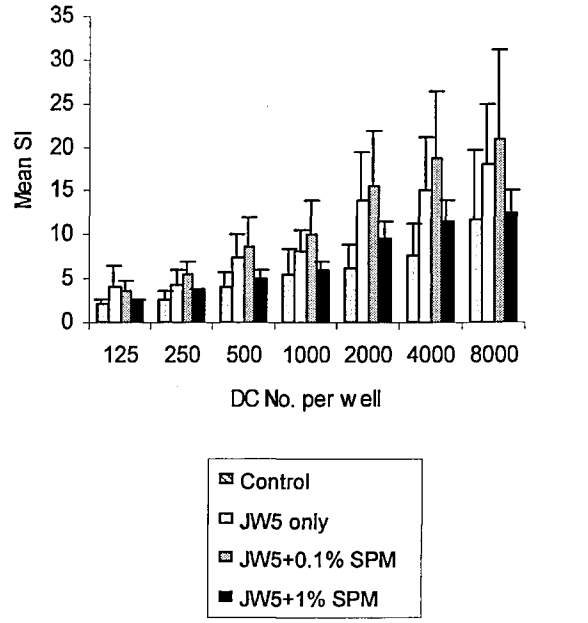
(b)



(c)



(d)



		125	250	500	1000	2000	4000	8000
P values (vs. control)	JW5 only	0.5	0.4	0.3	0.5	0.3	0.4	0.7
	JW5+0.1% SPM	0.4	0.2	0.3	0.4	0.2	0.3	0.6
	JW5+1% SPM	0.7	0.4	0.6	0.8	0.4	0.4	0.9
P values (vs. JW5 only)	JW5+0.1% SPM	0.9	0.6	0.8	0.7	0.8	0.7	0.9
	JW5+1% SPM	0.6	0.6	0.4	0.5	0.5	0.6	0.6
P values (vs. JW5+0.1% SPM)	JW5+1% SPM	0.5	0.3	0.4	0.3	0.4	0.4	0.6

infection with PE106 as determined by PCR. The summary of all three experiments using MDDCs infected with PE106 virus (fig.V.7d) or using MDDCs infected with JW5 virus (fig.V.8d) showed the enhanced allostimulatory potentials of virus infected MDDCs and further enhanced with 0.1% seminal plasma. Furthermore, MDDCs exposed to both 1% seminal plasma and virus exhibited higher allostimulatory capacity compared with control MDDCs. Such enhancement was associated with infection of the cells with HIV although viral DNA level was considerably low as demonstrated by PCR (fig.V.3). A decreased allostimulatory capacity of MDDCs was demonstrated when cells were pulsed with 1% seminal plasma from a HIV^+ patient with viral load of 2,000 copies/ml (fig.V.9a; P=0.03). Seminal plasma from a patient with viral load of 68,000 copies/ml also decreased the allostimulatory function of MDDCs but not significantly (fig.V.9b, P=0.07).

These data together with data obtained from viral DNA assay suggest that allostimulatory capacity of MDDCs are associated with level of infection with HIV. The suppressive effect of seminal plasma was not observed when the cells were exposed to virus at the same time although viral DNA assay could not detect viral DNA in these cells suggesting that virus may be captured by MDDCs or may attach to the cells which can infect and hence activate allogeneic T cells. Alternatively a very low infection level of MDDCs by HIV may be sufficient to activate and mature MDDCs and hence induces an enhanced allostimulatory capacity of these cells.

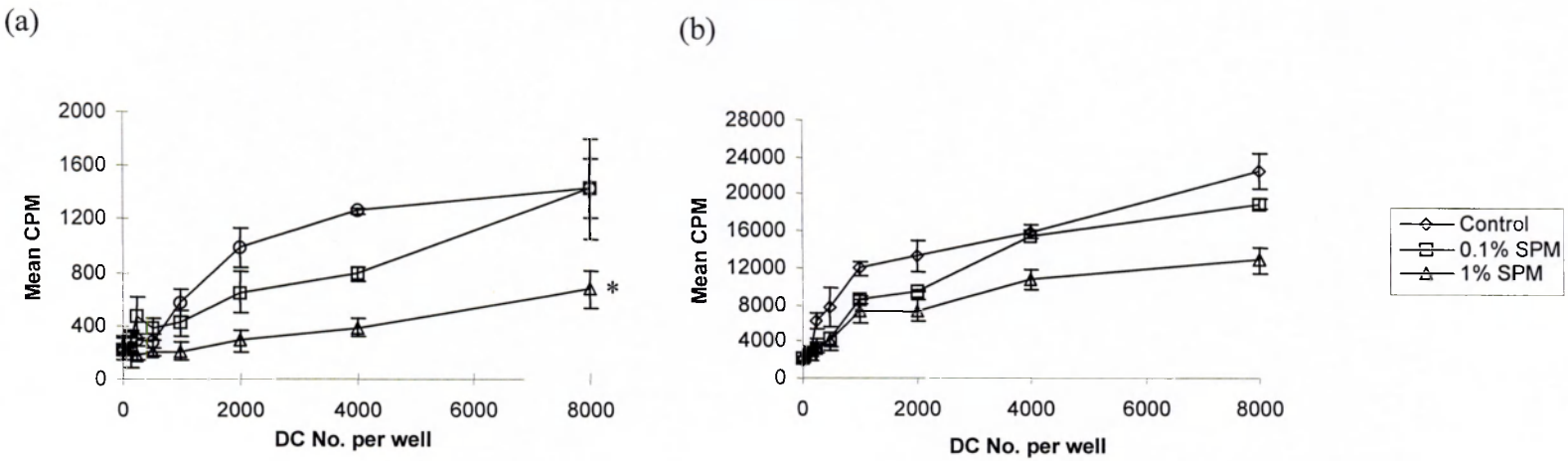


Fig.V.9. Allogeneic MLR with MDDCs pulsed with seminal plasma from HIV$^+$ patients

0.1% or 1% seminal plasma (SPM) from HIV$^+$ patients was added to the MDDC culture. Cells were washed next day and an allogeneic MLR was set up using 10^5 responder cells per well. Cells were pulsed with 0.5μCi of [^{3}H]-thymidine per well next day. The graphs show mean counts per minute (CPM) of triplicate with standard errors. (a) seminal plasma from patient 9 (viral load in seminal plasma was 2,000 copies/ml). (b) seminal plasma from patient 10 (viral load in seminal plasma was 68,000 copies/ml). Statistical analysis method employed was 'Independent sample T test' and were performed using a SPSS 10.0 software. P=0.03 (*) compared with allostimulatory capacity of control MDDCs (ie. untreated).

V.3. DISCUSSION

In this chapter the effects of seminal plasma on HIV-1 co-receptor expression on $CD14^+$ monocyte-derived dendritic cells, on HIV-1 infection of these cells, and on the antigen presentation function of HIV-1-infected MDDCs were investigated.

MDDCs expressed both primary HIV-1 co-receptors. Seminal plasma induced changes in surface expression of CCR5 and CXCR4. The level of CXCR4 expression on MDDCs was increased by seminal plasma whereas the level of CCR5 expression decreased (fig.V.2a-2d). This data suggests that seminal plasma may favour the infection of DCs with X4 strains of HIV-1. This was confirmed by *in vitro*-infection studies. Infection with X4 strain (JW5 virus; fig.V.3c) was only observed in 0.1% seminal plasma-pulsed MDDCs and infection with the R5/X4 strain [PE106 virus (fig.V.3b)] was enhanced when 0.1% seminal plasma was present. On the other hand infection with R5 strain (JRCSF) was detected in both untreated and 0.1% seminal plasma-pulsed MDDCs (fig.V.3b), but not in MDDCs pulsed with 1% seminal plasma. Although the level of CXCR4 expression on 1% seminal plasma-pulsed MDDCs, which was higher than on untreated MDDCs, was not significantly decreased compared with that on 0.1% seminal plasma no detectable level of infection of MDDCs by JW5 virus was observed. This suggests that seminal plasma may also inhibit entry of virus without decreasing CXCR4 expression. Alternatively such a small decrease may be sufficient to stop the X4 strains of virus entry. Our data, however, does not support the observation that R5

strains of virus are transmitted preferentially via sexual contact. It is possible that LC in the mucosa may not be the first target of viral infection as reported by others previously (Zhang *et al*, 1999).

Despite the fact that the same amount of virus (1ng/ml of p24 protein, 10^6 MDDCs/ml) was used to infect MDDCs, JRCSF virus infected MDDCs more efficiently than PE106 as was demonstrated by viral DNA assays [fig.V.3a and fig.V.3b, Lane B (virus only)]. This may be because 'dual' tropic strains like PE106 virus are actually mixed populations of both R5 and X4 quasispecies and PE106 may contain less of the R5 strain. Seminal plasma samples from two HIV^+ patients were also used to infect MDDCs and the viral DNA assay showed that these cells were readily infected with virus present in seminal plasma (fig.V.4). Furthermore, as little as 20 copies of virus were sufficient to infect DCs.

To determine whether DCs were productively infected with virus, p24 protein production by MDDCs infected with HIV-1 *in vitro* (table V.3) was measured by ELISA. Levels of p24 antigen in culture supernatants from allogeneic MLRs which were set up using HIV-1 infected MDDCs [infected with JRCSF, PE106 and JW5 viruses with or without seminal plasma (table V.2), and infected with virus present in seminal plasma from patient 10 (table V.3)] were also measured by ELISA to investigate whether productive infection occurred within $CD4^+$ T cells to which MDDCs transmitted virus. Antigen presenting function of HIV-1-infected MDDCs was assessed by allogeneic MLRs.

Little or no p24 protein release was detected by HIV-1 infected MDDCs, demonstrating that DCs may not be productively infected or the assay employed was not sensitive enough. Taken together with data from viral DNA assay it suggests that virus may be present in MDDCs in the latent form and consequently not available for infection of $CD4^+$ T cells. Whether DCs are productively infected with HIV is controversial. Several groups have shown that DCs are productively infected as has been demonstrated by electron microscopy showing budding of viral particles (Patterson *et al*, 1987; Blauvelt *et al*, 1997) and by detection of p24 protein (Chougnet *et al*, 1999; Kacani *et al*, 1998; Blauvelt *et al*, 1997). Indirect evidence has also been provided by the fact that DCs have been shown to transmit virus to monocytes (Kacani *et al*, 1998), and to T cells (Frank *et al*, 1999; Frankel *et al*, 1998; Ludewig *et al*, 1996). In contrast, others have detected entry of HIV but little evidence for productive infection in DCs without T cells (Granelli-Piperno *et al*, 1998; Weissman *et al*, 1995[a]; Weissman *et al*, 1995[b]; Ayehunie *et al*, 1995; Ramazzotti *et al*, 1995; Cameron *et al*, 1994; Pinchuk *et al*, 1994; Pope *et al*, 1994; Cameron *et al*, 1992). These latter results support our findings presented in this chapter where p24 antigen in MDDC supernatants was undetectable by ELISA. However, the DC-T cell association does provide a site of HIV replication, and the degree of infection appears to be related to the degree of T cell activation (Blauvelt *et al*, 1997; Cameron *et al*, 1994; Pinchuk *et al*, 1994; Tsunetsugu-Yokota *et al*, 1997). The exact mechanisms involved in the transmission of HIV from DCs to T cells are not fully understood. It has been suggested that CD40-CD40L [CD154] and CD28-CD80 interactions are important in this process (Pinchuk *et al*, 1994; Caux *et al*, 1994) suggesting

the importance of T cell activation. It has also been shown that direct contact of HIV-1-infected DCs with T cells through adhesion molecules, is crucial for efficient virus transmission from DCs to T cells and subsequent virus production (Tsunetsugu-Yokota *et al*, 1997). Whether MDDCs transmitted the virus to T cells was not determined by viral DNA PCR in this study. Supernatants from allogeneic MLR did not contain detectable levels of p24 antigens. There are several possible explanations as to why we were unable to detect p24 antigen released in DC supernatants or in allogeneic MLR supernatants in this study and they include:

i) The p24 protein ELISA used in this study may not be sensitive enough to detect p24 protein released. The detection limit of our assay ranged between 0.0316ng/ml and 1ng/ml. Others have shown that p24 production by DCs infected with HIV-1 without T cells or those co-cultured with T cells could be as little as 0.01ng/ml (Kacani *et al*, 1998; Blauvelt *et al*, 1997).

ii) The culture conditions/periods of cultivation employed in this study may not be optimum. Productive infection of DCs depends on the culture conditions as has been shown by the fact that when DCs were infected and cultivated without cytokine supplementation p24 antigen production was rarely observed whereas when cultivated in the presence of GM-CSF and IL-4 large amounts of p24 protein were produced after 7 days of cultivation (Kacani *et al*, 1998; Blauvelt *et al*, 1997). We have supplemented the cultures with GM-CSF and IL-4 every other day and have cultivated the cells for up to 5 or 6 days after incubation with virus overnight. Detectable levels of p24 were obtained

towards end of cultivation period [after 5 or 6 days of cultivation (table V.1)]. Therefore, cultivation time may not have been long enough to release larger amount of p24 and the system may have required a longer cultivation time.

iii) 1ng/ml (p24 protein concentration) of virus may not be sufficient to infect 10^6 DCs/ml productively. We did not evaluate the infectious dose [50% tissue culture infective dose ($TCID_{50}$)] of virus used to infect DCs. It is difficult to assess $TCID_{50}$ of JRCSF virus since $TCID_{50}$ are evaluated based on syncytium formation. JRCSF virus is R5 strain of virus and does not cause formation of syncitia (Dr. P. Hayes, personal communication). Thus it is difficult to conclude that the amount of virus used in this study was sufficient for detectable level of p24 proteins to be produced.

MDDCs which were infected with virus showed increased allostimulatory capacity. MDDCs infected with JRCSF virus or PE106 virus displayed higher allostimulatory capacity than MDDCs infected with JW5 (fig.V.5) and this observation was associated with levels of infection with the virus as demonstrated by PCR (fig.V.3). The suppressive effect of seminal plasma on MDDC function (chapter IV) was largely overcome if virus was present in the culture (fig.V.6-fig.V.8) although there was a decrease when MDDCs were exposed to 1% seminal plasma from HIV^+ patients (fig.V.9), compared with control MDDCs which were not exposed to seminal plasma. It has been shown that HIV-1 infected DCs may have increased pro-inflammatory activity as has been demonstrated by elevated production of TNF-α and IL-1β (Lore *et al*,

1999) as a result of activation of NF-κB (Kobayash *et al*, 1989; Okamoto *et al*, 1989). Therefore, it is possible that MDDCs infected with HIV in our study, may have produced TNF-α which abrogated the suppressive effect of seminal plasma on the allostimulatory function of MDDCs which was demonstrated in chapter IV. This Taken together, HIV exposure clearly overcame the suppressive effect of seminal plasma. However, productive infection of T cells was not demonstrated by p24 release, despite demonstration of virus entry and reverse transcription of viral RNA to DNA by PCR.

Future work

All the further work listed below could be performed with five populations of DCs described below:

i) DCs infected with HIV-1 alone.

ii) DCs infected with HIV-1 in the presence of TNF-α.

iii) DCs infected with HIV-1 in the presence of seminal plasma from HIV-1 negative individuals.

iv) DCs infected with HIV-1 in the presence of seminal plasma from HIV-1 negative individuals plus TNF-α.

v) DCs infected with virus present in seminal plasma from HIV^+ individuals.

1. <u>Do DCs become productively infected with HIV-1?</u>

Whether DCs are productively infected with virus should be measured using a more sensitive ELISA system (i.e. commercially available ELISA kits) for p24

antigen. Cultivation time with virus could be extended to allow release of more p24 proteins.

2. Can infected DCs activate T cells?

It has been shown that the degree of T cell infection parallels the degree of T cell activation (Cameron *et al*, 1994; Pinchuk *et al*, 1994; Tsunetsugu-yokota *et al*, 1997; Blauvelt *et al*, 1997). It has been suggested that infection with HIV results in a defect in antigen presenting function of DCs (Knight *et al*, 1993; Macatonia *et al*, 1989). Our data from allogeneic MLRs using HIV-infected MDDCs demonstrated that HIV infection of MDDCs did not result in a suppressed allostimulatory function. This could be confirmed by performing the following experiments: DCs could be co-cultured with $CD4^+$ T cells [both autologous and allogeneic] or recall antigen stimulated autologous $CD4^+$ T cells. The function of DCs could then be assessed by [^{3}H]-tymidine uptake.

3. Can infected DCs transmit virus to $CD4^+$ T cells?

After pulsing of DCs with virus, DCs could be co-cultured with autologous $CD4^+$ T cells for a longer period (up to 14 days). p24 antigen levels could be measured in culture supernatants collected at different time points and viral DNA could be assessed in cells which are harvested at different time points. To do this experiment sorting of the cells ($CD3^+$ cell sorting to eliminate DCs) prior to viral DNA assay would be required to differentiate infection of DCs from infection of T cells. This would allow investigation of whether DCs transmit virus to $CD4^+$ cell and also to monitor productive infection of these cells.

4. Quantitation of viral DNA in DCs.

Whether seminal plasma induces preferential infection with certain types of virus [R5, X4 or R5/X4 strains] could be determined by quantitating viral DNA copies of each virus in DCs after *in vitro* infection. Virus DNA could be quantitated using a LightCycler™ (Roche, Lewes, East Sussex, U.K.) which allows measurement of copy numbers.

5. Does viral load in semen have an influence on the suppressive effect of seminal plasma?

Our preliminary data showed that the viral load in seminal plasma appears to be correlated with a reduced suppressive effect of seminal plasma on the allostimulatory capacity of DCs. To confirm this phenomenon, the same experiment could be performed using larger numbers of HIV^+ seminal plasma samples with wide ranges of viral load. Also, *in vitro*-infection studies with different viral concentrations can be performed.

There are other factors which may have effect on the allostimulatory function of DCs. They include levels of TNF-α production which is probably elevated upon infection with HIV. The results from the study using HIV^+ seminal plasma did not correlate with the *in vitro*-studies and this may be due to different levels of and types of cytokines present in HIV^+ seminal plasma from HIV-negative seminal plasma and consequently results in a different outcome.

Since the development of a vaccine to prevent sexual transmission of HIV which is the most frequent mode of transmission, is the ultimate goal, the mechanisms of sexual transmission of HIV must be understood. Several different approaches have been used to elucidate mechanisms of sexual transmission of HIV such as SIV-macaque model (Hu *et al*, 2000; Miller and Hu, 1999; Joag *et al*, 1997; Blauvelt *et al*, 1997; Spira *et al*, 1996; Zambruno *et al*, 1995), skin explants (Blauvelt *et al*, 2000; Reece *et al*, 1998), and mucosa biopsies (Greenhead *et al*, 2000; Collins *et al,* 2000; Hu *et al*, 2000). Studies using mucosal tissues include the use of gastrointestinal biopsies (Olsson *et al*, 2000; Poles *et al*, 2000), rectosigmoidal biopsies (Olsson *et al*, 2000; Poles *et al*, 2000) and female reproductive tract biopsies (Collins *et al*, 2000; Greenhead *et al*, 2000). However, the histologic anatomy of these different mucosal tissues is very diverse. The nature and localisation of APCs which are suggested to be the first targets of HIV infection or association and overall structural cellular organisation differ in these tissues. Therefore, the most appropriate model to investigate the mechanisms of heterosexual transmission of HIV would be use of female genital tract tissues. *Ex vivo* studies together with *in vitro* studies described in this chapter would allow better understanding of the mechanisms of heterosexual transmission of HIV.

VI

DISCUSSION:

ROLE OF SEMINAL FLUID IN SEXUAL TRANSMISSION OF HIV-1

HIV-1 is transmitted vertically from mother to child, by sexual contact or by contact with infected blood. Sexual transmission is responsible for more than 90% of HIV-1 infections in Africa and in developing countries. The UNAIDS figures estimate that 5.3 million people were newly infected with HIV with 3 million deaths from AIDS occurred during the year 2000. This thesis focuses on factors influencing HIV-1 transmission via sexual contact. Semen is the vehicle for the virus during the sexual transmission of HIV from a male. Mucosal DCs are considered to play an important role in establishing infection and mediating the systemic spread of HIV due to their following characteristics: i) location within the mucosal epithelium, ii) expression of CD4 and CCR5 (Zhang *et al*, 1998; Zoeteweij *et al*, 1998; Zaitseva *et al*, 1997; Granelli-Piperno *et al*, 1996), iii) the ability to migrate to T cell-rich areas of lymph nodes (Hu *et al*, 2000; Banchereau *et al,* 1998) and iv) the ability to induce clustering, activation and transmission of HIV to T cells (Zoeteweij *et al*, 1998; *Blauvelt et al*, 1997). Therefore, this thesis examined, in particular, the main reservoir of HIV-1 in semen and the effect of seminal plasma on DC function, phenotype and susceptibility to HIV-1 infection.

VI.1. SUMMARY OF THESIS

The HIV-1 reservoir in semen was defined in order to evaluate 'sperm-washing' as a method of reducing the risk of HIV-1 transmission in HIV-discordant couples who wish to have children (chapter III). There is still controversy surrounding whether spermatozoa express CD4 and are infected or infectable with HIV-1. The study described in chapter III demonstrated that spermatozoa are not infected with HIV-1 *in vivo* as was demonstrated by the fact that neither viral RNA nor DNA was detected in these cells (Kim *et al*, 1999). The lack of potential for these cells to become infected was confirmed by phenotypic analysis of sperm cells. Spermatozoa did not express CD4 or CCR5 on the cell surface. Although there was a low level of CXCR4 expressed on spermatozoa, these cells lacked CD4 expression both at the protein and mRNA levels, suggesting that spermatozoa are not likely to be infectable with HIV.

Galactosylceramide (or a derivative) which has shown to be an essential component of the neural receptor for HIV-1 (Bath *et al*, 1991; Harouse *et al*, 1991), has been shown to be present on the surface membrane of the mid-piece and equatorial segments of human spermatozoa (Baccetti *et al*, 1994; Brogi *et al*, 1995). Although there is one report showing that sperm glycolipids are capable of binding to gp120 (Brogi *et al*, 1995), whether these glycolipids function as an alternative receptor for HIV infection of spermatozoa has not yet been confirmed. Since the mid-piece of sperm contains mitochondria that

generate energy for motility of sperm, it is possible that, if glycolipids on the surface of the mid-piece of sperm function as an alternative receptor for HIV, HIV infection may result in disrupted mitochondrial function and sperm immotility. This may explain an observation of Scofield *et al* in 1994 that HIV was detected in immotile but not in motile spermatozoa. 'Sperm-washing' involves isolation of motile sperm cells by a 'swim-up' procedure which would reduce or possibly even eliminate contamination with 'infected' non-motile sperm cells if these exist. Therefore, even with the assumption that sperm cells may be infected with HIV via glycolipids on the cell surface which may impair the motility of sperm cells, 'sperm washing' would still reduce the risk of transmission in HIV-discordant couples. It should be noted that in this study, virus was not detected in the less motile or dead sperm cell fraction. Recently, it has been shown that hepatitis C virus transmission can be reduced or eliminated by the 'sperm-washing' procedure (Pasquier *et al*, 2000). However, it has been suggested that hepatitis C virus is a blood-born infection and is not transmitted sexually (Dr. J. Gilmour, personal communication). Technical differences in HIV-1 detection and sperm preparation methods, or contamination of spermatozoal preparations with NSCs, may have resulted in discrepancies in results obtained by different groups.

HIV-1 was present in semen both as free-virus and NSC-associated virus (Kim *et al*, 1999) and these data have been supported by others (Pasquier *et al*, 2000; Coombs *et al*, 1998; Gupta *et al*, 1997; Dyer *et al*, 1996; Vernazza *et al*, 1996; Liuzzi *et al*, 1996; Rasheed *et al*, 1995). The present study also provided

indirect evidence that semen may be a distinct reservoir of HIV-1 as was demonstrated by the finding that levels of HIV-1 viraemia in semen did not always correspond with that in blood plasma or with blood CD4 counts. Other groups have reported similar findings (Coombs *et al*, 1998; Gupta *et al*, 1997; Liuzzi *et al*, 1996; Rasheed *et al*, 1995).

NSCs consist of immature germ cells, epithelial cells and leukocytes including macrophages, $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes and $CD103^+$ T cells (Quayle *et al*, 1997). It has been shown that among NSCs, T lymphocytes and macrophages are infected with HIV-1 *in vivo* (Quyale *et al*, 1997). The study described in chapter III showed that up to 45% of the NSC population express CD4, suggesting that these cells may be susceptible to HIV-1 infection. The mechanisms for preferential transmission of R5 strains and the initial events in infection after mucosal exposure to virus are not yet well understood. Mucosal integrity obviously plays an important role in sexual transmission of diseases. Due to their anatomical location, the vagina and ectocervix are the female genital tissues facing the potentially highest exposure to pathogens. These tissues are covered by layers of epithelial cells and antimicrobial secretions which provide a protective barrier. T cells (both $CD4^+$ T cells and $CD8^+$ T cells), B cells, Langerhans' cells and plasma cells are present in the female genital mucosa, located predominantly in the epithelium, parabasal and basal layers of the vagina and ectocervix (Johansson *et al*, 1999; Pope *et al*, 1998; Morris *et al*, 1983). It has been suggested that mucosal DCs may be the first cells to become infected following exposure to HIV (Miller and Hu, 1999;

Spira *et al*, 1996; Joag *et al*, 1997; Reece *et al*, 1998). Freshly isolated LCs (from skin) express functional CCR5 but not CXCR4 (Zaitseva *et al*, 1997) suggesting that selection of R5 strains could take place in the mucosa. Conversely, it has been shown that DCs isolated from human cervicovagina mucosa express CXCR4 but not CCR5 (Hladik *et al*, 1999). T cells derived from the human cervicovagina, form stable conjugates with DCs *in vitro* and result in productive infection by viruses of both co-receptor specificities (Hladik *et al*, 1999), suggesting that selection of virus variants does not occur by differential expression of co-receptors on genital cells. It has also been suggested that T cells in the endocervix may be the first cells to become infected (Zhang *et al*, 1999). Infection of T cells in the macaque endocervix with SIV was detected 3 days post inoculation. However, the possible infection of endocervical DCs or macrophages with SIV at day 3 was not assessed and therefore, it can not be concluded that T cells rather than DCs are the first cells to become infected. SIV infection was not detected in any cell type studied (i.e. T cells, $CD4^+$ cells, macrophages and DCs) 1 day after inoculation, but by day 7 infection of endocervical DCs and macrophages, in addition to T cells was detected (Zhang *et al*, 1999). Genotypic and phenotypic analyses of viral isolates from blood and semen (Zhu *et al*, 1996; Kroodsma *et al*, 1994) provided evidence that the semen may be a reservoir for R5 strains of virus thus allowing preferential transmission of R5 strains. The present study supports this hypothesis. It has been demonstrated that NSCs express CCR5, but negligible amounts of CXCR4, suggesting that this may contribute to the selection of R5 strains of virus within semen (chapter III). However whether

such low levels of CXCR4 expression on NSCs is sufficient to allow entry of X4 strains remains to be determined. Therefore, it is possible that R5 strain selection may take place in semen as well as in LCs at the mucosal surface.

In chapter IV the effects of seminal plasma on the phenotype and function of DCs were investigated. Immature DCs derived from peripheral blood $CD14^+$ monocytes using GM-CSF and IL-4 were generated as previously described (Sallusto *et al*, 1995; Sallusto and Lanzavecchia, 1994). Seminal plasma had suppressive effects on the allostimulatory capacity of DCs and reduced expression of co-stimulatory molecules on these cells (chapter IV). Whether such suppressive effects on allostimulatory capacity were the result of prostaglandin activity was investigated, since PGE2 has been shown to have immunomodulatory effects and is also known to be present in human semen at a concentration 10^8 times that seen in peripheral blood (Kelly, 1997[a]). PGE2 has both pro-inflammatory (Rieser *et al*, 1998; Reiser *et al*, 1997; Portanova *et al*, 1996; Kalinski *et al*, 1998) and suppressive effects (Strassman *et al*, 1994) on APCs. PGE2 inhibits IL-12 production in DCs (Kelly *et al*, 1997[b]; Kraan *et al*, 1995; Van Parijs *et al*, 1997) resulting in induction of type-2 polarised effector DCs (Kalinski *et al*, 1998; Kalinski *et al*, 1997) which induce Th-2 type responses (Kalinski *et al*, 1998). PGE2 has also been shown to stimulate IL-10 production in DCs (Groux *et al*, 1996; Kelly *et al*, 1997[b]) resulting in tolerogenic DCs, also known as type-3 polarised effector DCs (Steinbrink *et al*, 1997). These tolerogenic DCs express low levels of co-stimulatory molecules (Kalinski *et al*, 1998; Steinbrink *et al*, 1997) and hence may induce

anergy. The present data did not demonstrate a suppressive effect of prostaglandins on DC function. However, this study demonstrated that the suppressive effect induced by seminal plasma was abrogated by removal of lipids (chapter IV), suggesting that glycolipids in seminal plasma do have suppressive effects on DC function. It is possible that other components of seminal plasma may also play a role in induction of the suppressive effects, such as prostasome, polyamine, TGF-β and IL-10 although IL-10 is present in semen at very low concentrations (Kelly, 1995; Alexander and Ansderson, 1985). Although prostaglandin by itself did not induce suppressive effects *in vitro*, it is possible that prostaglandins may have DC suppressive effects in combination with other seminal components. It is also possible that spermine oxidation products resulting from an interaction with bovine serum may have resulted in the suppressed allogeneic MLRs as was seen in the murine MLRs (Labib and Tomasi, 1981). A further study using spermine oxidase inhibitors or pulsing DCs with seminal plasma in the absence of bovine serum in the DC medium would determine whether spermine oxidation products are responsible for the suppressed allogeneic T cell proliferation described in chapter IV. The possibility that the suppressive effect of seminal plasma might be due to induction of T cell anergy was investigated, but no such phenomenon was demonstrated (chapter IV).

The suppressive effect of seminal plasma on the allostimulatory function of DCs was overcome by addition of TNF-α (chapter IV). The decrease in expression of co-stimulatory molecules on DCs exposed to seminal plasma

was no longer observed when DCs were exposed to both TNF-α and to seminal plasma in combination. Previously published data have demonstrated that pro-inflammatory cytokines induce maturation of DCs (Sallusto and Lanzavecchia, 1997; Jonuleit *et al*, 1997; Cella *et al*, 1997; Roake *et al*, 1995; Sallusto *et al*, 1995). HIV was shown to infect DCs *in vitro* and this infection also abrogated the suppressive effect of seminal plasma on DCs (chapter V). This may result from maturation of DCs on antigen-uptake and/or increased production of pro-inflammatory cytokines (e.g. TNF-α and IL-1β) by HIV-1 infected DCs (Lore *et al*, 1999).

The expression of HIV-1 co-receptors on DCs was influenced by seminal plasma, by TNF-α and by both in combination (chapter V). TNF-α alone, seminal plasma alone or in combination induced a decrease in CCR5 expression and an increase in CXCR4 (chapter V) which may favour infection by X4 strains of HIV-1. These data were supported by those of others (Canque *et al*, 1999; Sallusto *et al*, 1998; Zaitseva *et al*, 1997). Expression of CXCR4 is also up-regulated by type-2 cytokines such as IL-4 and down-regulated by type-1 cytokines such as IFN-γ (Zoeteweij *et al*, 1998). Therefore, an increase in type 2 cytokine production, which can occur during HIV infection (Stylianou *et al*, 1999; Meroni *et al*, 1996) and elevated levels of pro-inflammatory cytokines such as TNF-α, IL–8 and IL-6, which can also occur in STDs, (Ramsey *et al*, 1995) may promote infection of DCs with X4 strains. The infection of seminal plasma-pulsed DCs, but not of control DCs, with an X4 strain of virus was observed (chapter V). Increased expression of CXCR4

on seminal plasma-pulsed DCs may result from TGF-β present in semen. TGF-β has been shown to up–regulate CXCR4 expression on DCs (Zoeteweji *et al*, 1998).

There are limitations in our study. $CD14^+$ monocyte-derived DCs were used as a mucosal LC model which has been found recently to be not an ideal model in terms of phenotype (fig.I.6). LCs derived from $CD14^-CD1a^+$ LC precursors, LCs isolated from female mucosa, or mucosal biopsis obtained from the female genital tract would be a better model. A summary of the thesis is outlined in fig.VI.1. It also describes proposed mechanisms which may be important in the suppressive effects of seminal plasma on DCs which would provide 'protection' to the 'male cells' in a 'female environment', and mechanisms where 'danger' which would be apparent if a pathogen was present, may be tackled by functioning DCs.

VI.2. SIGNIFICANCE OF THE *IN VITRO* FINDINGS TO THE *IN VIVO* SITUATION

VI.2.1. The effect of seminal plasma and TNF-α on DC function in the female reproductive tract

The function of the DC in mucosal tissues, including the female reproductive tract, is to take up and process antigen of potential pathogens and present peptides of the antigen in association with MHC molecules. The presented antigen may then be recognised by both naive and memory T cells, resulting in

T cell activation via co-stimulatory molecules expressed on the DC surface (including CD40, CD80 and CD86) and ultimately leading to the generation of an immune response to the pathogen. This sequence of events involves both maturation and migration of the DC from that of an antigen uptake phenotype at the mucosal epithelium to that of an immunostimulatory phenotype in the local lymph node, where the vastly higher T cell density facilitates interaction of the DC with the T cell.

The mucosa of female reproductive tract, like other mucosa, encounters many potential infectious agents and consequently an effective barrier must be presented. The vagina and ectocervix, which are potentially the tissues facing the greatest exposure to pathogens, including HIV-1 in semen, are covered with layers of epithelial cells (fig.I.4) and antimicrobial secretions such as lactoferrin, lysozyme and transmembrane mucins (e.g. Mucin 1 or episialin) and secretory mucins (DeSouza *et al*, 1999; Gendler and Spicer, 1995). Such anatomy provides a primary protective barrier. Although potential pathogens must be eliminated, the main function of the female reproductive tract is to facilitate insemination, fertilisation and development of the embryo. To the female, cells in semen are allogeneic, however to promote fertilisation of the embryo an immune response to spermatozoa should be avoided. The results of this and other studies demonstrate some of the mechanisms that humans have evolved to both promote fertilisation by minimising immune responses to seminal components but also discriminate and respond to potential pathogens if present within the semen. The significance of the present findings on these

two opposing functions (fertilisation versus response to pathogens) can best be explained by describing the possible *in vivo* situations. In this study, TNF-α was employed as an inflammatory stimulus, which would be one inflammatory component present in semen when a male is infected with a pathogen (STDs) *in vivo*.

i) Exposure of the female reproductive tract to semen in the absence of inflammation

Exposure of MDDCs to seminal plasma would mimic the *in vivo* situation where the female reproductive tract would be exposed to semen in the absence of an inflammatory stimulus or STD. In this situation mucosal epithelial DC (i.e. LC) function would be suppressed by seminal plasma as demonstrated by decreased expression of co-stimulatory molecules, along with a decreased ability to stimulate T cells. Therefore, an immune response to spermatozoa would be discouraged, promoting fertilisation. This inhibition of an immune response to semen may be more important in the long term, with the female potentially exposed frequently to semen over her entire reproductive life-span and beyond. Seminal plasma appears to discourage an immune response by affecting DC rather than T cell function directly as seminal plasma pulsed MDDCs did not appear to induce anergy in T cells. T cells were not exposed directly to seminal plasma as the accepted model of DC function is that DC-T cell interaction occurs in the lymph node away from contact with seminal plasma. T cells exposed to seminal plasma pulsed MDDCs proliferated in response to IL-2 and to freshly added seminal plasma unpulsed MDDCs. The

present study demonstrated that lipids might be the major suppressive component of seminal plasma. However, individual lipid molecules may activate DC function demonstrating that the suppressive effects are induced by a complex interaction between seminal components.

ii) Exposure of the female reproductive tract to an inflammatory stimulus (without semen)

Exposure of MDDCs to TNF-α alone would mimic the *in vivo* situation where the female reproductive tract would be exposed to an inflammatory stimulus or STD in the absence of semen. This situation may occur either as a result of a non-sexually transmitted vaginal infection (e.g. thrush) or due to a STD that may establish itself at a time point subsequent to insemination. The results of the present study demonstrated that under these conditions mucosal DC function would be induced, with maximal increases in expression of co-stimulatory molecules and T cell stimulatory abilities. Such factors would promote an immune response to the pathogen.

iii) Exposure of the female reproductive tract to semen in the presence of inflammation

Exposure of MDDCs to seminal plasma and TNF-α would mimic the *in vivo* situation where the female reproductive tract would be exposed to semen in the presence of an inflammatory stimulus or STD. Some suppressive effect of seminal plasma was evident in this study as the increases in expression of CD86 and allostimulatory abilities were not as great as in MDDCs exposed to

TNF-α alone. However, the present data would suggest that in this circumstance mucosal DC function would be retained, with increased ability to stimulate T cells, compared with unexposed mucosal DCs. Therefore, an immune response to a possible pathogen would be promoted.

VI.2.2. The relationship between DCs in the female reproductive tract and HIV-1

The interaction between seminal plasma, TNFα, DCs and HIV-1 was investigated in order to elucidate factors that may influence sexual transmission of HIV-1 to the female with the focus being the potential for the DC to be infected with HIV-1.

i) Influence of seminal plasma and TNF-α on CCR5 and CXCR4 expression by MDDCs

HIV co-receptor expression was assessed as described in chapter IV Exposure of MDDCs to seminal plasma, TNF-α or both together resulted in decreased expression of CCR5 but increased expression of CXCR4. Therefore, if DCs are infected with HIV-1, exposure to seminal plasma and/or TNF-α may promote infection with X4 rather than R5 strains. However, CCR5 and CXCR4 expression would have evolved not to allow infection with HIV-1, but rather to mediate cellular responses to the chemokines RANTES, MIP-1α, MIP-1β and SDF-1. One of the main functional attributes of a DC is that of migration from the mucosal tissue to the lymph node, with chemokines

controlling this process. Therefore, decreased CCR5 but increased CXCR4 expression may represent a migratory DC phenotype, reducing the ability to respond to RANTES, MIP-1α and MIP-1β produced in the mucosal tissue, especially in inflammation, but increasing the ability to migrate towards SDF-1 present in the lymph node at higher concentrations (Delgado *et al*, 1998; Sallusto *et al*, 1998).

The finding that either seminal plasma or TNF-α alone induced a migratory phenotype in the DC is in contrast to the effect of these two agents on DC co-stimulatory abilities, with seminal plasma suppressing and TNF-α enhancing such activities. Therefore, exposure of DCs to seminal plasma components promotes maturation of DCs in terms of migratory abilities but without increased T cell stimulatory abilities.

ii) HIV-1 infection of DCs and the influence of seminal plasma

The present study assessed the susceptibility of MDDCs to infection with R5 (JRCSF), X4 (JW5) and dual tropic (PE106) strains of HIV-1. In addition the effect of seminal plasma on MDDC susceptibility to infection was assessed, thereby mimicking the *in vivo*-situation were the mucosal epithelium of the female reproductive tract would be exposed to HIV-1 in the presence of seminal plasma.

Detection of viral DNA in HIV-1 exposed MDDCs demonstrated infection of these cells with all three strains of virus. However, no evidence of productive

infection could be demonstrated by release of p24 protein into MDDC culture supernatants. Together, these findings suggest that HIV-1 viral particles may attach to DCs via CD4 and either CCR5 or CXCR4 co-receptors and fuse with the cellular membrane. Viral RNA is then reverse transcribed into viral DNA, which was detected by PCR. Whether viral DNA may then integrate into the DC genome, forming stable proviral DNA, was not assessed. The lack of productive infection and evidence from other studies would argue against this (Granelli-Piperno *et al*, 1998; Weissman *et al*, 1995[a]; Weissman *et al*, 1995[b]; Ayehunie *et al*, 1995; Ramazzotti *et al*, 1995; Cameron *et al*, 1994; Pinchuk *et al*, 1994; Pope *et al*, 1994; Cameron *et al*, 1992). Studies of HIV-1 replication in T cells have demonstrated that an activated host cell is required for productive infection (Blauvelt *et al*, 1997; Cameron *et al*, 1994; Pinchuk *et al*, 1994; Tsunetsugu-Yokota *et al*, 1995). HIV-1 may enter resting T cells, with reverse transcription of viral RNA, however, in resting T cells viral DNA remains in an unstable unintegrated form without productive infection. Stable integration of proviral DNA in to the T cell chromosome requires T cell activation. However, integration of proviral HIV DNA does occur in terminally differentiated macrophages (Wienberg *et al*, 1991; Lewis *et al*, 1992) and therefore, HIV-1 may well integrate into terminally differentiated DCs.

Seminal plasma exposure resulted in prominent alterations in the susceptibility of MDDCs to the three different strains of HIV-1, as demonstrated by PCR, which appeared to reflect the seminal plasma induced alterations in co-receptor

expression by MDDCs. The absence of seminal plasma favoured infection of MDDCs with the R5 strain JRCSF, with these cells displaying a $CCR5^{high}$ / $CXCR4^{low}$ phenotype. Seminal plasma pulsed MDDCs were susceptible to the X4 strain JW5, with these cells displaying a $CCR5^{low}$ / $CXCR4^{high}$ phenotype. Both unpulsed and seminal plasma-pulsed MDDCs were susceptible to infection with the dual tropic strain PE106.

Therefore, seminal plasma favoured infection of DCs with X4 strains of HIV-1 apparently due to increased expression of CXCR4 and decreased expression of CCR5. In addition, seminal plasma exposure may also affect the function of these co-receptors on the DC as well as their level of expression, however this was not assessed in the present study. Semen would be present at the point of insemination and therefore, the results of this study suggest that infection of DCs in the epithelium of the female reproductive tract with X4 strains of HIV-1 would be favoured if this strain of virus is present in semen. This is contrary to the compelling epidemiological evidence the R5 strain is the predominant HIV strain transmitted. This evidence would argue against direct infection of the DC, with viral fusion and reverse transcription of RNA, being the key event in transmission of HIV-1. However it is possible that semen may be a reservoir for R5 strains of virus due to the high level of CCR5 expression (but only low levels of CXCR4 expression) on NSCs (Kim *et al*, 1999) with the R5 strains predominating in semen (Zhu *et al*, 1996; Kroodsma *et al*, 1994).

HIV-1 exposed MDDCs displayed increased T cell stimulatory abilities as shown by their increased allostimulatory capacities. As was the case for TNF-α, HIV-1 infection overcame the suppressive effects of seminal plasma, with MDDCs exposed to both virus and seminal plasma displaying greater T cell stimulatory capacity than untreated MDDCs. This increased T cell stimulatory ability was greatest with each viral strain with the corresponding seminal plasma concentration favouring DC infection.

VI.3. THE ROLE OF MUCOSAL EPITHELIAL DCS IN SEXUAL TRANSMISSION OF HIV

Using the MDDC as a model for immature DCs, the present study investigated the possible interactions between mucosal DCs in the female reproductive tract, seminal plasma, inflammatory stimuli and HIV-1. The ultimate aim was to elucidate the possible mechanisms of and factors influencing sexual transmission of HIV-1. In relation to HIV transmission the present study has demonstrated that:

ii) Seminal plasma favoured infection of DCs with X4 strains of HIV-1.

iii) Seminal plasma had suppressive effects on DC co-stimulatory cell surface phenotype and T cell stimulatory ability.

iv) This suppressive effect was overcome either by exposure to an inflammatory stimulus (TNF-α) or to HIV-1 itself.

v) Seminal plasma or TNF-α induced a $CCR5^{low}/CXCR4^{high}$ migratory phenotype.

The direct involvement of the DC being a key event in transmission of HIV-1 remains unproven. However, data from this and other studies demonstrates that the biology of the DCs may result in this cell playing a crucial role in HIV-1 transmission. Using the data of this study as the focus, but with data from other studies included, the following sequence of events and a possible role of epithelial mucosal DCs in HIV-1 transmission to the female is proposed:

Step 1) Sexual exposure of the female to semen from a HIV^+ man.

DCs in the mucosal epithelium may be exposed to both seminal plasma and to HIV-1, either as free virus in the seminal plasma or as infected NSCs. Epidemiological data suggests that impaired mucosal integrity due to the presence of ulcerative STDs increases the chance of sexual transmission of HIV-1. Such impaired mucosal integrity would increase the exposure of mucosal leukocytes, including the DC, to HIV and semen. STDs also increase numbers and / or activation status of DCs, macrophages and T cells which are susceptible to viral infection.

Step 2) Association of HIV-1 with the DC

HIV-1 may then associate with the DCs *in vivo* in two ways and both may feasibly occur simultaneously. HIV may bind to the DCs via CD4 and either CCR5 or CXCR4 co-receptors, resulting in viral fusion and reverse

transcription of viral RNA but without productive infection. The presence of semen may favour infection of DCs by X4 strains of HIV-1. HIV-1 may also associate with the DC at the cell surface without fusion of the viral particle. A role for the adhesion molecule DC-SIGN in binding HIV at the DC surface has been suggested (Geitenbeek *et al*, 2000[b]). There is no evidence that semen affects the strain of HIV that may bind via DC-SIGN and presumably both R5 and X4 strains may bind in this manner, if present. However, other studies have shown that R5 strains predominate in semen (Zhu *et al*, 1996; Kroodsma *et al*, 1994) and the present study demonstrates CCR5 rather than CXCR4 expression by NSCs.

Step 3) Dissemination of HIV-1 via the DC

DCs exposed to semen would mature to a $CCR5^{low}$ / $CXCR4^{high}$ migratory phenotype and migrate towards the lymph node in response to a concentration gradient of SDF-1. TNF-α may also be present in semen, which may enhance the alteration to a migratory phenotype. The $CCR5^{low}$ phenotype may assist migration away from RANTES, MIP-1α and MIP-1β, should the mucosal epithelial region be inflamed. Therefore, HIV associated with the DC would be carried from the mucosal tissue to the lymph node where DCs would cluster with T cells, facilitating infection of T cells in the lymph node. It would be expected that virions associated with the DC surface via DC-SIGN would be the HIV particles transferred to the T cell. However, a low and undetectable level of productive HIV infection in DCs could conceivably result in T cell infection.

In the present study DCs exposed to and apparently infected with HIV-1, displayed increased T cell stimulatory abilities, even in the presence of suppressive seminal plasma. Exposure to seminal TNF-α would further enhance DC-T cell stimulatory abilities. Therefore, the DCs in contact with HIV-1 and responsible for HIV transmission to the T cells, would also be the DC with a greater ability to stimulate T cells. This enhanced T cell stimulation would further promote productive viral replication in the DC-T cell cluster with establishment of the infection in the lymph node and ultimate dissemination of HIV.

The mucosal tissue exposed to HIV and semen would contain leukocytes other than DCs that would be targets for more productive HIV replication. However, the marked ability of the DC, rather than other mucosal leukocytes, to migrate from the mucosal epithelium directly to the T cell rich lymph node, followed by DC-mediated T cell activation with productive viral replication, implicates the DC as an important factor in the sexual transmission of HIV-1 and subsequent systemic dissemination.

One of the desirable outcomes of an efficient treatment or vaccine for HIV, in addition to lowering blood plasma viral load, would be a lowering of the viral load in semen as semen serves as the primary vector for sexual transmission of HIV since it contains both cell-associated and cell-free viruses. In order to stop systemic spread of virus infection after genital exposure, a vaccine which can

induce potent immunologic memory cell populations that rapidly expand in response to the presence of HIV recall antigens within the genital tract or draining lymph nodes is desirable. Another important fact to be considered in order to reduce the incidence of sexual transmission of HIV is treatment of other STDs. This can both reduce viral shedding in semen and in vaginal fluid and can restore mucosal integrity, providing a more effective barrier to, and protection from HIV and other STDs.

Fig.IV.1. Proposed mechanisms for the suppressive effect of seminal plasma on DCs and for TNF-α–induced and/or virus-induced abrogation of the suppressive effect

1. Seminal plasma (SPM) suppressed allostimulatory ability of DCs significantly and such the suppressed allostimulatory capacity of DCs correlated with reduced expression of co-stimulatory molecules on these cells. Suppression may be due to induction of tolerogenic DCs (type-3 effector DCs) by activities of immunosuppressive components in semen such as 19-OH-PGE2, TGF-β, IL-10, and prostasome (Kalinski *et al*, 1998; Steinbrink *et al*, 1997).

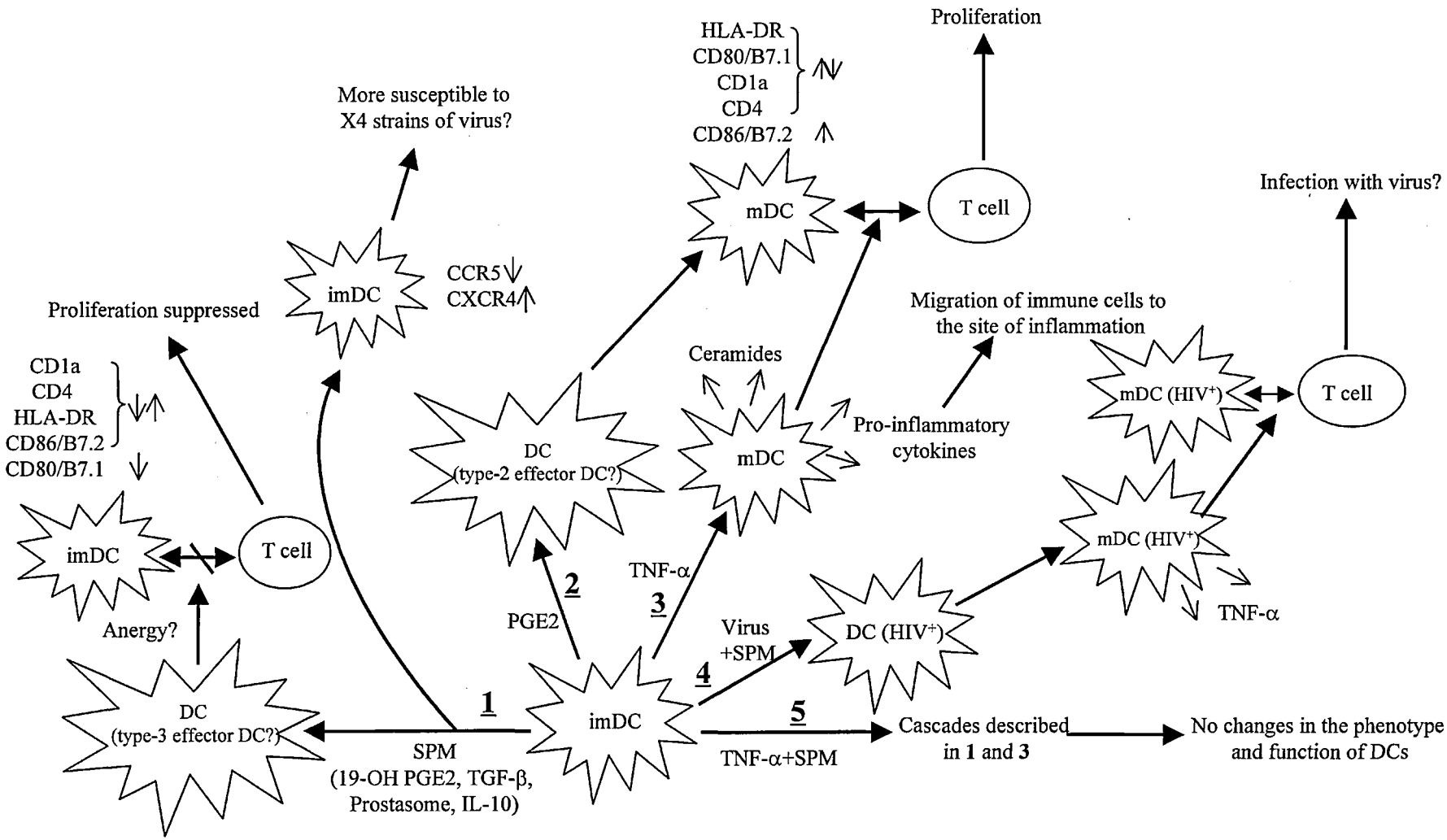
2. Exogenous PGE2 although lipids in seminal plasma play a role in the induction of the suppressive effects on the function of DCs, did not have the same effect on the function of DCs as seminal plasma. Exogenous PGE2 has shown to induce differentiation of immature DCs to type-2 effector DCs resulting in type 2 responses (Kalinski *et al*, 1998).

3. Recombinant TNF-α induced enhanced allostimulatory ability of DCs. TNF-α induces ceramide release in DCs resulting in inhibition of endocytosis (Sallusto and Lanzavecchia, 1997; Jonuleit *et al*, 1997) and also induces secretion of pro-inflammatory cytokines by DCs as a result of activation of NF-κB (Kobayashi *et al*, 1989; Okamoto *et al*, 1989)

4. When virus was present in culture the suppressive effect of seminal plasma was abrogated. Infection with HIV induces release of TNF-α which may induce migration of other immune cells to the site of inflammation *in vivo* and may also induce further maturation of DCs themselves.

5. TNF-α blocked the suppressive effect of seminal plasma and *vice versa*. TNF-a may overcome the suppressive effect of seminal plasma by inducing maturation of DCs as described in 3, however seminal plasma also counteracts with the effect of TNF-a. Thus, this counterbalancing may have resulted in unchanged phenotype and function of DCs.

(imDC: immature DC; mDC: mature DC; ⟶: induction; ⟷: interaction; ⇒: secretion; ↑: increase; ↓: decrease; ⇵: no change


HLA-DR
CD80/B7.1
CD1a
CD4
CD86/B7.2
Proliferation
More susceptible to
X4 strains of virus?
mDC
T cell
Infection with virus?
CCR5
CXCR4
imDC
Proliferation suppressed
Migration of immune cells to
the site of inflammation
Ceramides
CD1a
CD4
HLA-DR
CD86/B7.2
CD80/B7.1
DC
(type-2 effector DC?)
mDC
Pro-inflammatory
cytokines
mDC (HIV+)
T cell
imDC
T cell
TNF-α
2
3
PGE2
Virus
+SPM
4
DC (HIV+)
mDC (HIV+)
Anergy?
TNF-α
DC
(type-3 effector DC?)
1
imDC
5
Cascades described
in 1 and 3
No changes in the phenotype
and function of DCs
SPM
(19-OH PGE2, TGF-β,
Prostasome, IL-10)
TNF-α+SPM

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